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Modulation of alternative splicing regulators during epithelial-mesenchymal transition in cancer progression

MODULATION OF ALTERNATIVE SPLICING REGULATORS DURING EPITHELIAL-MESENCHYMAL TRANSITION IN CANCER PROGRESSION

By

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ABSTRACT

The epithelial-mesenchymal transition (EMT), one of the hallmarks of cancer, is a set of biochemical changes that allow epithelial cells to lose their polarization to transform into mobile mesenchymal cells. Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and 2) are RNA-binding proteins that act as splice factors and regulate a large number of transcripts that promote epithelial characteristics in the cell. Among the genes regulated by ESRPs, fibroblast growth factor receptor 2 (*FGFR2*) is well-known that has two mutually exclusive exons switching in the EMT. ESRPs were identified as key regulators of EMT during cancer progress. The aim of my project is to reveal ESRPs' role in prostate cancer.

To explore ESRPs' functions in tumours, I constructed stable PC3 cell lines overexpressing either ESRP1, or ESRP2, or both ESRP1 and 2. These cells were injected in nude mice subcutaneously to grow xenografts, and tumour sizes were measured by calliper. Functional properties of these cells such as cell growth, migration rate, and EMT properties were also measured.

As ESRPs regulate splicing of FGFR2 in EMT, I have used a biochromatic reporter whose fluorescent output is dependent on the inclusion/exclusion of *FGFR2* exon IIIc. Compounds from the LOPAC library were screened using HEK293 cells with the reporter to find hits that promote exclusion of exon IIIc in *FGFR2* splicing and possibly block EMT. Several assays were carried out including cell growth assay, EMT markers staining, cell migration and proliferation assays *in vitro*. While *in vivo*, xenografts were developed by injecting PC3 cells in nude mice subcutaneously, and then treated with one of the hit compounds to explore whether it could affect tumour growth.

Three chemicals were selected that switch splicing of *FGFR2*, verified as modulators of EMT by increasing E-cadherin expression and junctional localization, showed different activities in cell functional assays *in vitro*, and one of them significantly decreased tumour growth *in vivo* in prostate cancer xenografts mouse models. I have also found that ESRPs overexpression may suppress tumour growth *in vivo* in prostate cancer xenografts mouse models.

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I would also like to say thanks to my parents, sisters, wonderful friends and my husband for supporting me spiritually throughout writing this thesis and my life in general.

AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: ....

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ABBREVIATIONS

3’ UTR	3’ untranslated region
5’ UTR	5’ untranslated region
AJ	adherens junctions
Akt	v-akt murine thymoma viral oncogene homolog 1
ANOVA	analysis of variance
APCs	antigen-presenting cells
AS	alternative splicing
AUF1	ARE/poly(U)-binding/degradation factor 1
bHLH	basic helix-loop-helix
BM	basement membrane
BMP	bone morphogenetic protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CELF	CUG-BP, Elav-like family
CTBP	C-terminal binding protein
CUGBP2	CUG triplet repeat, RNA binding protein 2
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRED	discosoma red fluorescent protein
DSS	distal splice site

E-cad	E-cadherin
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EMT	epithelial mesenchymal transition
ERK	extracellular regulated kinase, mitogen activated protein kinase
ESE	exonic splicing enhancer
ESRP	epithelial splicing regulatory protein
ESS	exonic splicing silencer
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FKBP12	12-kDa FK506-binding protein
FBS	fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FOX1/2	RNA binding protein, fox-1 homolog 1/2
FTDP	frontotemporal dementia with Parkinsonism
GPCR	G protein coupled receptor
GSK3β	glycogen synthase kinase-3β
GATA	transcription factors bind to the DNA sequence "GATA"
HEK293	human embryonic kidney cell line
HIF	Hypoxia-Inducible Factor
HIFU	high-intensity focused ultrasound
hnRNP	heterogeneous ribonucleoprotein particle
HRP	horseradish peroxidase

HuR	human antigen R, also known as ELVAL1
ID	inhibitor of differentiation proteins
IGF	insulin-like growth factor
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JNK	c-Jun N-terminal kinases
KOR	κ-opioid receptors
LNCaP	lymph node carcinoma of the prostate cell line
LOPAC	library of pharmacologically active compounds
LPA	lysophosphatidic acid
M-MLV RT	Moloney murine leukaemia virus reverse transcriptase
MAPK	mitogen-activated protein kinase
MAPT	microtubule-associated protein tau
MOI	multiplicity of infection
MOR	μ-opioid receptors
mRNA	messenger RNA
NMD	nonsense mediated decay
NSCLC	non-small-cell lung cancer
PARP	poly ADP ribose polymerase
PBMCs	peripheral-blood mononuclear cells
PBS	phosphate-buffered saline
PC3	prostate cancer cell line
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor

PDK1	3-phosphoinositide-dependent protein kinase-1
PDX	Patient-Derived Xenograft
PFA	paraformaldehyde
PI3K	phosphatidylinositol-4, 5-bisphosphate 3-kinase
PSS	proximal splice site
PTB	polypyrimidine tract-binding protein
PTC	premature termination codon
PVDF	polyvinylidene fluoride
Rac	Ras-related C3 Botulinum Toxin Substrate
RBM	RNA Binding Motif Protein
RIPA	radio immunoprecipitation assay buffer
RNA	ribose nucleic acid
RNAi	RNA interference
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SAPK	stress- activated protein kinase
SIN1	stress- activated protein kinase interacting protein 1
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLUG	snail family transcriptional repressor 2 (also called SNAI2)
siRNA	small interfering RNA
SMA	spinal muscular atrophy
SMAD	homologues of the Drosophila protein
SMN2	survival motor neuron 2
SNAI1	snail family transcriptional repressor 1 (also called SNAIL)
snRNP	small nuclear ribonucleoprotein particle

SRN	splicing regulatory network
SR protein	serine/arginine rich protein
SRPK	serine/arginine protein kinase
SRSF	serine/arginine-rich splicing factor
TAK1	Transforming growth factor beta-activated kinase 1
TGFβ	transforming growth factor β
TJ	tight junctions
TNFα	tumour necrosis factor α
TTP	Tristetraprolin, also known as zinc finger protein 36 homolog [ZFP36]
TWIST1/2	Twist related protein 1/2
TβRI/II	TGF-β type I receptor, also known as TGFR1/2
UV	ultraviolet light
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

Chapter 1 Introduction

1.1 Prostate cancer

Prostate cancer (PCa) is a heterogeneous disease and the most commonly diagnosed non-cutaneous malignancy in men (Cozar et al., 2018; Gatta et al., 2013; Shoag & Barbieri, 2016). Currently, PCa is the second most common diagnosed cancer among men and has the fifth highest death rate (Stewart BW, 2014). PCa has a high widespread presence with a relatively low cancer death (Hema, Thambiraj, & Shankaran, 2018; Moghadam, Taheri, & Peiravian, 2018).

Most cases of PCa can be safely followed by active surveillance or observation. The current treatment options such as surgery, hormone therapy, chemotherapy, and radiation therapy are insufficient due to the heterogeneity of this cancer and this has spurred demand for improved technologies (Hema et al., 2018). Androgen-deprivation therapy (ADT) is a typical treatment of advanced or metastatic PCa that is related to reduction of bone mineral density (BMD) and increasing frequency of osteoporotic fractures (Campos et al., 2018).

1.1.1 *Prostate cancer in Western world*

According to statistics from the US Centres for Disease Control, the incidence of PCa in African Americans is highest, followed by Europeans. The lowest incidence of PCa is in Asian ethnic groups. In the United States, the five-year survival rate for PCa patients is approximately 98.2% (Institute, 2018). PCa is the second highest diagnosed frequency rate in men and has the fifth highest cancer mortality (BW & CP, 2014). In 2012, about 1.1 million men suffered from PCa, and 307,000 died from PCa in the world (BW & CP, 2014). PCa is the most common form of cancer in men in 84 countries (BW & CP, 2014), and it is more common in developed countries, but the prevalence in developing countries is increasing (Baade, Youlden, & Krnjacki, 2009). Due to the promotion of PSA testing, the discovery rate of PCa in the 1980s and 1990s increased significantly (Stewart BW, 2014). Autopsy studies have shown almost 30% to 70% of men over the age of 60

who did not die of PCa had histological evidence of PCa (Welch & Black, 2010; Zlotta et al., 2013).

According to a report by Prostate Cancer UK (https://prostatecanceruk.org), over 47,000 men are diagnosed with PCa and more than 11,000 men die from PCa every year in the UK. PCa is seen predominantly in men over 50 years old and frequency increases with age. Men diagnosed with PCa are usually between 65 and 69 years old.

1.1.2 *Signalling pathways in Prostate Cancer*

The occurrence and development of PCa is characterised by a series of complicated processes. The abnormality of intracellular signal pathways plays one of the central roles of PCa progression. It has been shown in several reports that growth factors contribute to the progress of PCa since they either raise the steroidal hormone level directly or increase enzyme effectiveness indirectly. Various studies have revealed that Hedgehog signalling pathway, Wnt signalling pathway, Notch signalling pathway and mitogen-activated protein kinase (MAPK) signalling pathway play a crucial role in the occurrence, invasion and metastasis of PCa. (Joshi et al., 2015). It was found by Kypta and Waxman that the Wnt/β-catenin pathway played a special role on tumour cells invasion by affecting cell growth, cell variation and the EMT in prostate cancer (Kypta & Waxman, 2012). Nguyen et al. presented some evidence concerning the role of the NF-κB pathway in PCa and discussed therapeutic challenges to target the NF-κB pathways. They also indicated that it is essential to further analyse inflammatory pathways in PCa in order to develop applicable preventive methods and design novel therapeutic approaches (Nguyen, Li, Yadav, & Tewari, 2014). It was revealed that for a normal prostate, the Notch signalling pathway was essential, while its dysfunction could promote PCa progression (Villaronga, Bevan, & Belandia, 2008).

1.1.3 *Genetic events underlying prostate cancer*

Prostate cancer has a high degree of heterogeneity. Driver mutations contributing to cancer development can have high frequency, low frequency, or be rare. Driver mutations are mostly identified by their frequencies. Thus, high-frequency drivers are identified; but rare drivers may not be. Recently, in order to find oncogenic drivers with

low-frequency mutations that were not identified yet, researchers have collected whole-genome exon sequencing data reported in recent years and identified 97 significant mutations. Of the 97 potential prostate cancer oncogenes, 70 are new genes with no reports to have been previously linked to cancer development and progression in prostate cancer. By comparing the genomes of castration-resistant prostate cancer and primary cancer, they confirmed that most of the oncogenic mutations are concentrated in signal pathways such as AR, WNT / β-catenin, PI3K, RAS-MAPK, and DNA repair (Armenia et al., 2018). Consistently, molecular biology studies of prostate cancer have shown that a variety of oncogenes and tumour suppressor genes are involved in the occurrence and development of prostate cancer, and their related genes (such as *p53, BCL-2, PTEN*, etc.) and the mechanism of prostate cancer initiation and the evaluation of prognosis is closely related (G. C. Wang, Zhao, Spring, & DePinho, 2018). In addition, some apoptosis-related genes and DNA repair genes are also new targets for molecular genetics and gene therapy.

One of the causes of malignant tumours is the disruption of the dynamic balance of apoptosis. Among the regulators of apoptosis, BCL-2 is one of the most important suppressors. BCL-2 overexpression occurs in patients with highly malignant prostate cancer and is related to androgen resistance and resistance to anticancer drugs (Catz & Johnson, 2003; Lin, Fukuchi, Hiipakka, Kokontis, & Xiang, 2007). The research by Bubendorf et al. showed that the prognosis of BCL-2 negative prostate cancer is much better than that of BCL-2 positive, and the expression of BCL-2 protein in prostate cancer tissues is positively correlated with pathological grade (Bubendorf et al., 1996). Other studies have shown that the expression of BCL-2 reduces the chances of patients becoming resistant to multiple anticancer drugs during chemotherapy, making tumour cells sensitive to cytotoxic drugs. BCL-XL can inhibit the apoptosis effect of cytotoxic drugs (Chaudhary, Abel, & Lalani, 1999).

Mutations of the tumour suppressor gene *p53* (also called TP53) are common in prostate cancer, and the expression of the *p53* protein is related to high Gleason grade, pathological stage, and localized prostate cancer hyperplasia. *p53* gene responds to diverse cellular stresses to regulate expression of genes involved in cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism in prostate cancer. p53

protein expression can be used as an independent prognostic indicator of prostate cancer and as a guide to treatment. It has been observed in studies of advanced prostate cancer that as the tumour progresses, the P53 expression level increases and reaches the highest level in tissues from patients with androgen tolerance (Barbieri et al., 2012; Beltran et al., 2016; G. C. Wang et al., 2018).

In 1997, Li et al. and Steck et al. reported a tumor suppressor gene on chromosome 10q23.3, called *PTEN*, independently of each other (J. Li et al., 1997; Steck et al., 1997). *PTEN* is the first tumour suppressor gene with phosphatase activity discovered to date. It does not only participate in cell cycle regulation, but also suppresses the PI3K-AKT-mTOR pathway to regulate cell survival, proliferation and energy metabolism (Barbieri et al., 2012; Grasso et al., 2012). *PTEN* is the most widely mutated tumour suppressor gene in prostate cancer. The loss of heterozygosity at this site occurs in 40% of prostate cancers, and it rises to 60% in prostate cancer cell lines (Taylor et al., 2010; G. C. Wang et al., 2018). Mutations and reductions in *PTEN* may contribute to the distant metastatic potential of cancer cells, and often occur in lethal prostate cancer. Currently, the ability of *PTEN* to regulate the apoptosis of prostate cancer cells is an active area of research.

Genetic mutations in DNA repair genes are also quite common in metastatic prostate cancer, such as mutations in *BRCA2, ATM, CHEK2, BRCA1, RAD51D*, and *PALB2*. Studies show that more than 10% of men with aggressive prostate cancer carry genetic mutations in the DNA repair gene. This ratio is more than four times that of ordinary people and more than twice that of patients with localized prostate cancer. It is worth noting that patients with advanced prostate cancer are 18 times more likely to carry *BRCA2* mutations than other men. Anticancer drugs have been approved to treat prostate cancers carrying such mutations, such as PARP inhibitors or platinum drugs (Pritchard et al., 2016). Furthermore, Amplification, mutation and splicing variants of androgen receptor (AR) play an important role in the progression of prostate cancer and the resistance to ADT by allowing constitutive activation of the AR pathway (Heinlein & Chang, 2004; Okegawa et al., 2018).

The study of genetic events in prostate cancer is not only a useful supplement to molecular pathology research, but also provides a reliable basis for the prognosis of prostate cancer. In addition, it can also provide new treatment strategies for gene

therapy of prostate cancer, especially for androgen-independent prostate cancer. Due to the complexity of the pathogenesis and molecular biology of prostate cancer, genetic events related to prostate cancer still need to be explored.

1.1.4 *Diagnosis and treatment of PCa*

For PCa diagnosing, all the tests have their advantages and disadvantages. There are three main generally applied tests for PCa: blood tests, physical prostate check (known as a digital rectal examination or DRE) and biopsy. The blood test, also called a prostate-specific antigen (PSA) test, detecting the PSA level in blood is a useful tool for diagnosing early-stage PCa. It was shown that prostate-specific antigen had a correlation with raised tumour occurrences, while there was no evidence of a connection between it and death rate (Djulbegovic et al., 2010). PSA tests are not regular tests offered to screen for PCa due to the untrustworthiness of the results. In addition to PCa, the PSA level may also raise because of benign prostatic hyperplasia (BPH), an infection of the urinary tract or prostate inflammation, which is not related to carcinoma. Also, the levels of PSA do not correlate with the stage of PCa. As a consequence, an increased level of PSA may result in over-examination and therapy (https://prostatecanceruk.org/prostate-information/prostate-tests/psa-test).

Since most diagnosed PCas are asymptomatic, the US Preventive Medicine Task Force (USPSTF) does not recommend the use of PSA testing in order to prevent overdiagnosis and overtreatment. The USPSTF believes that the benefits of detection do not exceed the possible drawbacks (Fleshner, Carlsson, & Roobol, 2017; Moyer & Force, 2012).

The most commonly examination routines in hospital for PCa are DRE, serum PSA, transrectal ultrasound, and pelvic MRI. As it is quite frequent that a patient with PCa develops a bone metastasis, radionuclide bone scans are options available prior to the scheduled therapy. Confirmed PCa requires pathological examination by prostate biopsy.

5α-reductase inhibitors may reduce the risk of lower-grade PCa but have no correlation with high-risk PCa and are therefore not a good choice for the inhibition of PCa (Stewart BW, 2014). Supplements with minerals or vitamins have no effect on PCa risk (Stewart BW, 2014; Stratton & Godwin, 2011).

Radical treatment can be used for patients with early PCa. Radioactive particle implantation, radical prostatectomy, and radical external radiation therapy can be used to cure early PCa. Comprehensive treatment methods should be used for patients with intermediate PCa, such as surgery combined with radiotherapy, or endocrine therapy plus radiotherapy and so on.

PCa is a clinically heterogeneous disease (Cozar et al., 2018). Although some of the inert male diseases could be observed safely, be treated by local treatments, a major subgroup of these diseases will reappear and finally develop an aggressive, castration resistant PCa. The castration-resistant metastasis is the main reason for most cases of mortal PCa. (Caram, Estes, Griggs, Lin, & Mukherjee, 2018; Moghadam et al., 2018; Whang et al., 2013)

Endocrine therapy is the mainstay of hormone sensitive advanced PCa. Methods of endocrine therapy include castration (surgical castration or drug castration) and anti-androgen therapy (bicalutamide or flutamide) or castration + anti-androgen treatment (Moghadam et al., 2018; Okegawa et al., 2018; Wong, Ferraldeschi, Attard, & de Bono, 2014). The efficacy of surgical castration or drug castration is basically the same. However, almost all patients eventually develop hormone-independent PCa or hormone-resistant PCa. For patients with castration-resistant PCa, second-line endocrine therapy or new endocrine therapy (abiraterone, enzalutamide, etc.) can be used. (Armstrong et al., 2011; Caram et al., 2018; Lorch, 2017; Moghadam et al., 2018). Patients with hormone-refractory PCa should continue to maintain castration while using chemotherapy based on docetaxel and mitoxantrone (Campos et al., 2018; Lorch, 2017; Okegawa et al., 2018; Shoag & Barbieri, 2016). Patients with PCa with bone metastases should be treated with bone protectants (primarily bisphosphonates) to prevent and reduce bone-related events, relieve bone pain, improve quality of life, and improve survival. External radiation therapy or radionuclides can also improve local bone pain.

In the last few years there have been quite a few efforts to try to correlate PCa clinical appearances with genomic information (Cozar et al., 2018; Shoag & Barbieri, 2016). Based on this it is possible to find patterns of gene expression that correlate with various stages of PCa and they can be used as biomarkers, help in diagnosis or guide therapy

(Shoag & Barbieri, 2016). Over the last few years, it has also assisted us to gain more information about disease pathogenesis, treatment efficiency, and disease development.

Furthermore, the mechanism regulating the metabolism and growth of tumour cells is also an important means to controlling the proliferation and metastasis of prostate cancer cells. Recently, Andrea Alimonti's group from the Institute of Oncology Research, University of Italian-speaking Switzerland found a new mechanism regulating the metabolism and growth of tumour cells, suggesting that tumour cells can be "slimmed down" to achieve the effect of inhibiting tumour growth (J. J. Chen et al., 2018; M. Chen et al., 2018). The glucose uptake and metabolism of prostate cancer cells—Glycolysis activity—are increased relative to normal cells, but prostate cancer cells cannot maintain cell proliferation if they cannot synthesize enough lipids themselves. They detected a nearly 10-fold increase in pyruvate dehydrogenase complex (PDC) activity in prostate cancer cells relative to normal proliferating cells. The increase in PDC activity is an important reason for the synthesis and accumulation of lipids in prostate cancer cells.

A recent issue of Nature Genetics also published a study by Professor Pier Paolo Pandolfi of Harvard University on mouse PCa models (M. Chen et al., 2018). The results showed that high-fat foods may relate to PCa metastasis. In the mouse PCa model, a series of changes such as tumour suppressor gene mutation and enhanced lipid synthesis activity was observed before tumour metastasis. The drugs that Andrea Alimonti studies to block lipid synthesis in tumour cells also offer the possibility of preventing PCa metastasis.

Every type of PCa therapy method may result in serious side effects, like erectile dysfunction and the loss of bladder control. The latest therapies, for example, high-intensity focused ultrasound (HIFU) or cryotherapy, have been developed to avoid them.

Finally, in recent years, a new therapy has emerged - immunotherapy. Tumour immunotherapy is a treatment method to control and clear tumours by restarting and maintaining the immune system's recognition and killing of tumour cells and restoring the normal anti-tumour immune response of the body. Compared to traditional

treatment, immunotherapy acts not directly on cancer cells and tumours, but through the patient's immune system. This kind of therapy not only works well, but also avoids damage to the body, so it has received much attention in recent years. Immunomodulators are one of the most effective new classes of drugs in PCa immunotherapy (Alberti, 2016; Maia & Hansen, 2017).

Tasquinimod is an immunomodulatory drug that is crucial in modulation of myeloid cell function (Brower, 2016; Gupta, Al Ustwani, Shen, & Pili, 2014; Maia & Hansen, 2017; Silvestri et al., 2016). It has been revealed that Tasquinimod has a tumour suppression function in animal experiments of PCa and has been shown to be useful in clinical trials as well (Gupta et al., 2014). As a result of regulation of immune cells, immunosuppression and vessel growth in the tumour microenvironment was reduced and metastasis and spread was decreased (Shen et al., 2015). Indoximod, as a well-known methylated tryptophan blocker for the enzymatic indoleamine 2,3-dioxygenase (IDO) pathway - by tryptophan consumption, induces T cell dysfunction and increases immunosuppressive effects (Alberti, 2016). In contrast, in a certain number of Tregs, Indoximod can enhance the immune response to the Sipuleucel-T vaccine (a kind of cancer treatment vaccine (Kantoff et al., 2010)) in metastatic castration-resistant prostate cancer (mCRPC) patients (Jha et al., 2017; Kolawole, Hixon, Dameron, Chrisman, & Smirnov, 2015). Lenalidomide has been developed as the derivative of thalidomide that has fewer side effects. In addition to its anti-inflammatory and anti-angiogenic features, Lenalidomide also acts to enhance the immune response, which is why it is already used alone or in combination with chemotherapy drugs (especially docetaxel and paclitaxel), in trial mCRPC patients' therapy. Unfortunately, although it has moderate anti-tumour results, serious adverse reactions have also been revealed. (Nabhan et al., 2014; Quinn, Shore, Egawa, Gerritsen, & Fizazi, 2015; Yap et al., 2016).

1.2 Epithelial-mesenchymal transition

The epithelial-mesenchymal transition (EMT) was primarily observed and identified as a property of embryogenesis by Elizabeth Hay in the early 1980s (Hay, 1995). EMT involves in a biological process where epithelial cells transform into mesenchymal cells by a specific functional transition. EMT, and its reversal procedure, mesenchymal-epithelial transition (MET) are vital in organ development, chronic inflammation, tissue regeneration, cancer metastasis and various fibrotic diseases (T. Chen, You, Jiang, & Wang, 2017; Kalluri & Weinberg, 2009). Epithelial and mesenchymal cells have different features and functions, as well as several of the same properties (Lamouille, Xu, & Derynck, 2014).

The key scientific issues in research of the EMT mechanism in tumour metastasis are to illuminate the modulation of EMT in tumour cells, to reveal the pathology in the initiation, progression and metastasis of tumours, and to develop diagnostic techniques and therapeutic approaches targeting EMT key molecules. (Shapiro et al., 2011)

1.2.1 *Definition and mechanisms*

EMT, as a hallmark of cancer, is a set of phenotypical changes that occur when epithelial cells convert into mesenchymal stem cells (Ling & Oltean, 2017). Epithelial cells sit on a basement membrane (BM) and are linked with each other through junctions. There is a polarized distribution of cytoskeleton proteins, like actin, from apex to the base. In contrast, mesenchymal cells are low in polarity, shaped like a spindle and linked with each other only by the focal point (Kalluri & Weinberg, 2009) (Figure 1-1). By EMT, the epithelial cells lose the epithelial phenotype, through loss of cell polarization connectivity with the BM, and gain new properties to migrate and invade, escape from cell death and degrade cell skeleton proteins, acquiring morphological properties of mesenchymal cells.

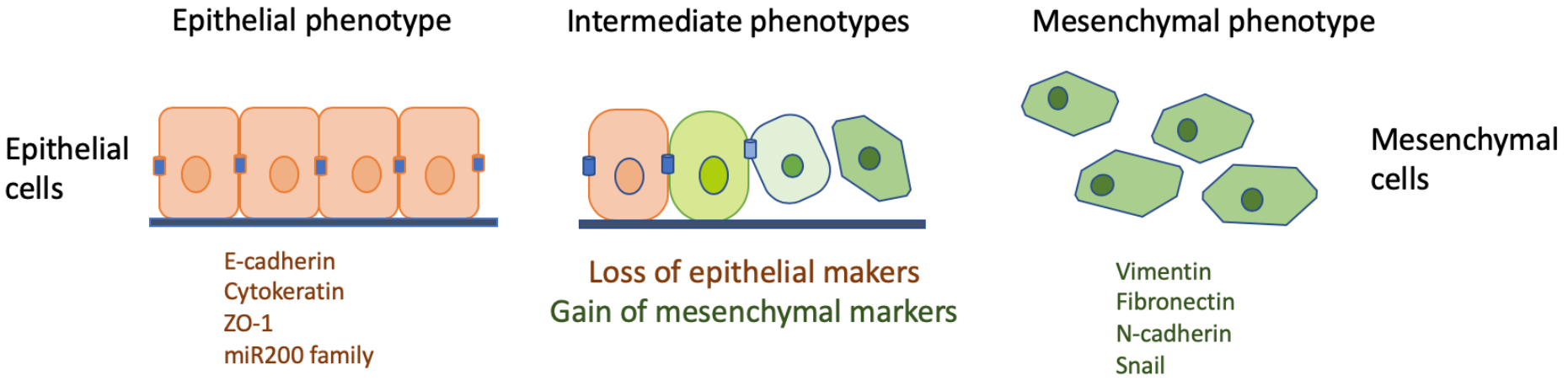


Figure 1-1 EMT process and the intermediate phenotypes as cell transition.

Some common epithelial and mesenchymal cell makers are listed: E-cadherin, Cytokeratin, ZO-1 and miR200 family for Epithelial cells and Vimentin, Fibronectin, N-cadherin and Snail for mesenchymal cells. Adapted from (Kalluri & Weinberg, 2009).

At the 2007 International EMT Conference in Portland and the 2008 Cold Spring Harbor Laboratory Meeting, EMT was categorized into 3 types on the basis of a specific biological environment in which EMT occurred: embryogenesis and organ development (Type I), organ fibrosis and tissue regeneration (Type II), and tumour progression (Type III) (Kalluri, 2009) (Figure 1-2). EMT associated with embryo implantation, development and organ formation is called “type I EMT”. Its main biological function is the generation of secondary epithelial cells through mesenchymal cell metaplasia (MET), which enables the diversification of cell types during embryogenesis. EMT occurring during injury repair, tissue regeneration and organ fibrosis is defined as “type II EMT”. Its main biological role is through the production of fibroblasts to repair tissue damage caused by traumatic and inflammatory reactions. Physiologically, the conversion stops spontaneously when the inflammatory response is relieved; however, as the inflammatory response continues to be activated, the EMT process will persist and eventually lead to organ fibrosis (Kalluri & Neilson, 2003). “Type III EMT” refers to phenotypic transformation associated with epithelial malignancies. Primary epithelial tumour cells form migratory mesenchymal cells via type III EMT, migrate to different sites with blood flow, and then form epithelial tumour metastases by the reverse MET process. Unlike mesenchymal cells that completely lose epithelial phenotypes formed by EMT type I and type II, metastatic tumour cells formed by type III EMT acquire mesenchymal phenotype while maintaining certain epithelial cell characteristics (T. Chen et al., 2017; Kalluri & Weinberg, 2009).

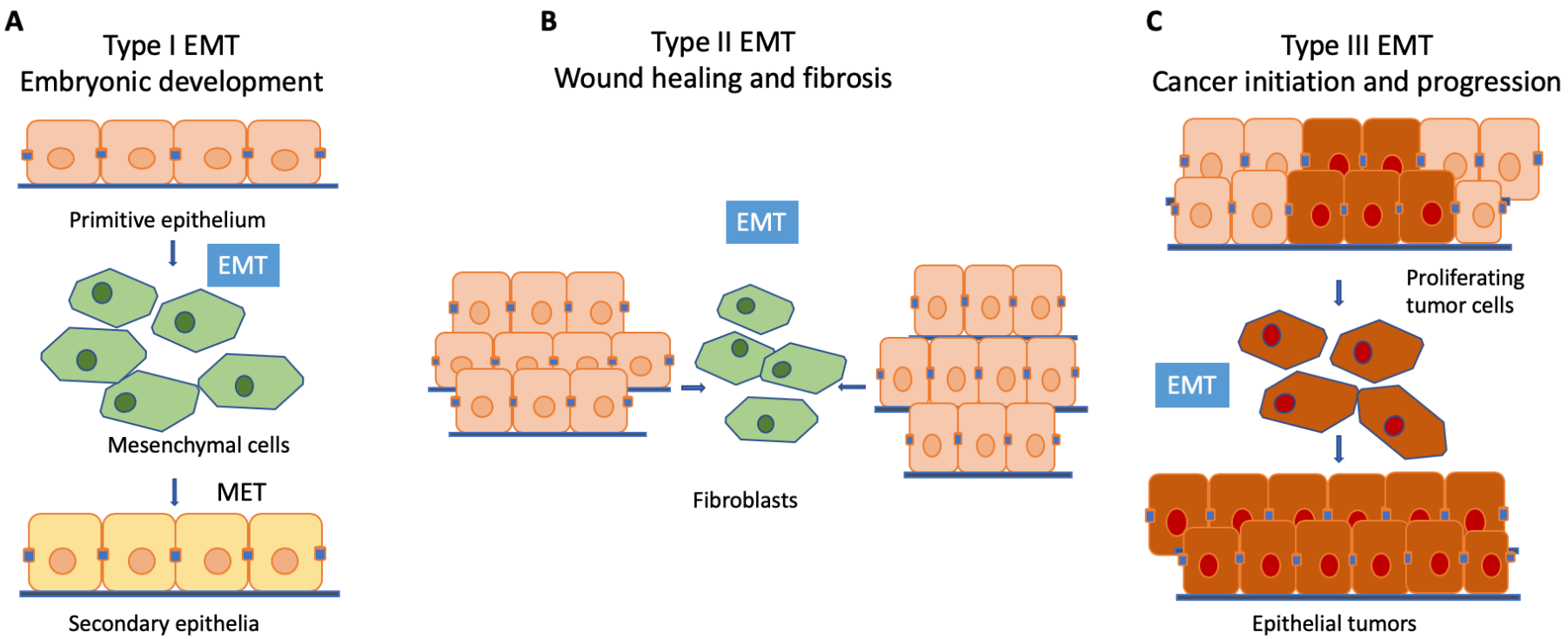


Figure 1-2 Classification of EMT into three different subtypes.

(A) *Type 1 EMT is related embryogenesis which is generally shut during adult life.* ***(B)*** *Type 2 EMT appears in wound healing and fibrosis* ***(C)*** *Finally, type 3 EMT is associated with the initiation of tumour progression, invasion and metastasis. Adapted from (Kalluri & Weinberg, 2009).*

EMT is controlled at several levels – post-translational level, transcriptional and post-transcriptional, differential splicing regulation and non-coding RNA level (De Craene & Berx, 2013) (Figure 1-3). Many transcription factors have been described as contributing to the regulation of EMT, including basic helix-loop-helix (bHLH, including TWIST1/2), snail family transcriptional repressor 2 (SLUG, also called SNAI2), snail family transcriptional repressor 1 (SNAI1, also called SNAIL), and Zinc finger E-box binding homeobox (ZEB). These transcription factors work together to regulate the initiation and process of EMT through different signalling pathways and interactions between pathways. Members of the Snail family encode transcription factors with a zinc finger structure, including SNAI1 (SNAIL), SNAI2 (SLUG), and SNAI3 (SMUC), where SNAI1 and SNAI2 are important in embryonic development (mesoderm, gastrulation, neural crest development), tissue fibrosis and the EMT process in tumours. Snail binds to the E-box DNA sequence and promotes EMT phenotypic changes by inhibiting epithelial gene expression (Brzozowa et al., 2015). It also disrupts intercellular interactions by inhibiting the expression of claudin and occludin which results in promoting the EMT process. In addition, receptor tyrosine kinases (RTKs), or glycogen synthase kinase-3β (GSK3β) can also affect the EMT process by regulating the expression of Snail in EMT-related pathways (Namba, 2015; Y. F. Wang, Shi, Chai, Ying, & Zhou, 2013).

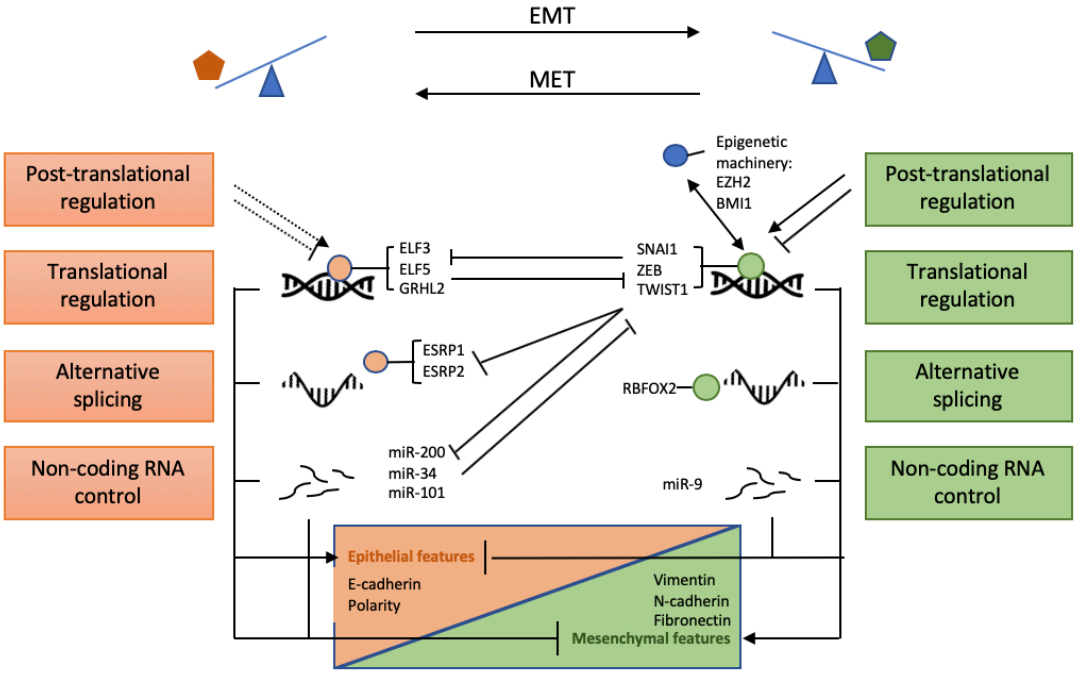


Figure 1-3 Regulatory interactions regulating EMT.

Some transcription factors inducing EMT, e.g. ZEB, TWIST1 and SNAI1, are essential in the regulation and collaboration with post-translational control. AS is demonstrated by specific splicing factors, like ESRPs and RBFOXs switch splicing towards a certain direction. However, non-coding RNAs also control EMT. EMT is instigated by the instability of the networking. Adapted from (De Craene & Berx, 2013)

ZEB mainly includes ZEB1 and ZEB2, which are important intranuclear transcription factors. The transcriptional repression function of ZEB is mainly accomplished with the assistance of C-terminal binding protein (CTBP). In addition, the expression of ZEB is usually accompanied by the activation of Snail. SNAI1 alone or in combination with TWIST1 can induce the expression of ZEB1 and promote the EMT process.(J. Xu, Lamouille, & Derynck, 2009)

In addition to the several transcription factors that are well-defined in the mechanisms mentioned above, other transcription factors also play a central role in regulating EMT. For example, forkhead box (FOX) with a winged helix and GATA transcription factor (described by their capability to bind to the DNA sequence "GATA") with a zinc finger structure can alter the polarity and connectivity of epithelial cells. The SOX (SRY box, Sex determining region Y boxes) can synergize with SNAI1 or SNAI2 to promote cell

metastasis and invasion (Guo et al., 2012). These transcription factors have been shown to have an influence on the EMT process, but the mechanism is not fully revealed.

Epigenetic modifications suppressing epithelial properties then improve the function of these TFs. Though the TFs expression level is crucial for EMT, there are several TFs controlled by post-translational regulation which is acting through their distribution and degradation in cells (Thiery, Acloque, Huang, & Nieto, 2009).

Adding to the direct effects of EMT-related transcription factors on gene expression, RNA splicing, and miRNA regulation can also have important effects on the structure and function of EMT-related proteins. Alternative splicing is a co-transcriptional process through which different mature RNAs may result and therefore different proteins. Several recently identified splicing regulatory proteins, such as ESRP1 and ESRP2, which define specific epithelial or mesenchymal splice variants are involved, adding a new level to the regulation network of EMT. Non-coding RNA regulation is also a master level that influences the cell type by repressing the genes modulating the epithelial and mesenchymal phenotype.

In embryogenesis, as well as in natural and converted cell lines, EMT is induced by numerous signalling pathways (Figure 1-4). Among these signalling pathways, some are activated by various proteins of the transforming growth factor-β (TGF-β) family: Notch, Wnts, epidermal growth factor (EGF), fibroblast growth factor (FGF), Hypoxia-Inducible Factor(HIF), Hepatocyte Growth Factor (HGF), and many others are included as well (Thiery et al., 2009).

TGF-β is an important cytokine that regulates EMT progression. The TGF-β superfamily consists of 33 members, including three TGF-β subtypes, two types of activins, and bone morphogenetic proteins (BMPs). It plays an important role in embryonic development, wound healing, tissue fibrosis and malignant tumour development.

TGF-β regulates EMT via the Smad pathway (Smad proteins: homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma). The TGF-β family member binds to the serine/threonine kinase receptor and phosphorylates the TGF-β type II receptor (TβRII, also known as TGFR2), which in turn activates the TGF-β type I receptor (TβRI, also known as TGFR1).

Afterwards TβRI phosphorylates downstream R-Smads (receptor-regulated Smads, Smad2 and Smad3); R-Smads binds to Smad4 to form a trimer complex. In BMP signalling, Smad1 and Smad5 act synergistically with Smad4 to activate or inhibit transcription of related genes by interacting with transcriptional coactivators or transcriptional co-inhibitors (Bosukonda & Carlson, 2017). In addition, Smads can directly activate the expression of some mesenchymal cell-related genes such as *VIM, FN* and *COL1A1*, regulating EMT progression (Heldin & Moustakas, 2012; Shin et al., 2011).

TGF-β can also regulate EMT through the non-Smad pathway. In the non-Smad pathway, TGF-β regulates EMT progression primarily through the Rho family, PI3K/Akt, and MAPK pathways in small G proteins. Activation of Rho, Rac (Ras-related C3 Botulinum Toxin Substrate) and other GTPases can induce actin recombination and formation of lamellipodia and filopodia (Zago, Biondini, Camonis, & Parrini, 2017; Zhou, Shi, Tian, Zhou, & Gao, 2016). Inhibition of Akt can inhibit the expression of SNAI1 and increase the expression of E-cadherin, thereby regulating the EMT process (X. L. Wang et al., 2016). In addition, TGF-β can also activate the extracellular regulated kinase (ERK), P38, and c-Jun N-terminal kinases (JNK)/MAPK pathways. For example, transforming growth factor beta-activated kinase 1 (TAK1) is an upstream kinase of the P38 MAPK and JNK pathways. When stimulated by TGF-β, it activates TAK1, thereby activating the p38 MAPK and JNK pathways (I. T. Chen, Hsu, Hsu, Chen, & Tseng, 2015). c-Jun is a component of the activator protein 1 (AP1) complex, which activates c-Jun and synergizes with Smad3-Smad4 to regulate TGF-β-induced transcription (Yi, Park, Jung, Jang, & Kim, 2015).

In addition to the TGF-β family, other growth factors are also significant in the EMT process. They mainly include FGF, HGF, insulin-like growth factor 1 (IGF1), EGF, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), etc., which can induce epithelial cell phenotype to stromal cell phenotype transformation.

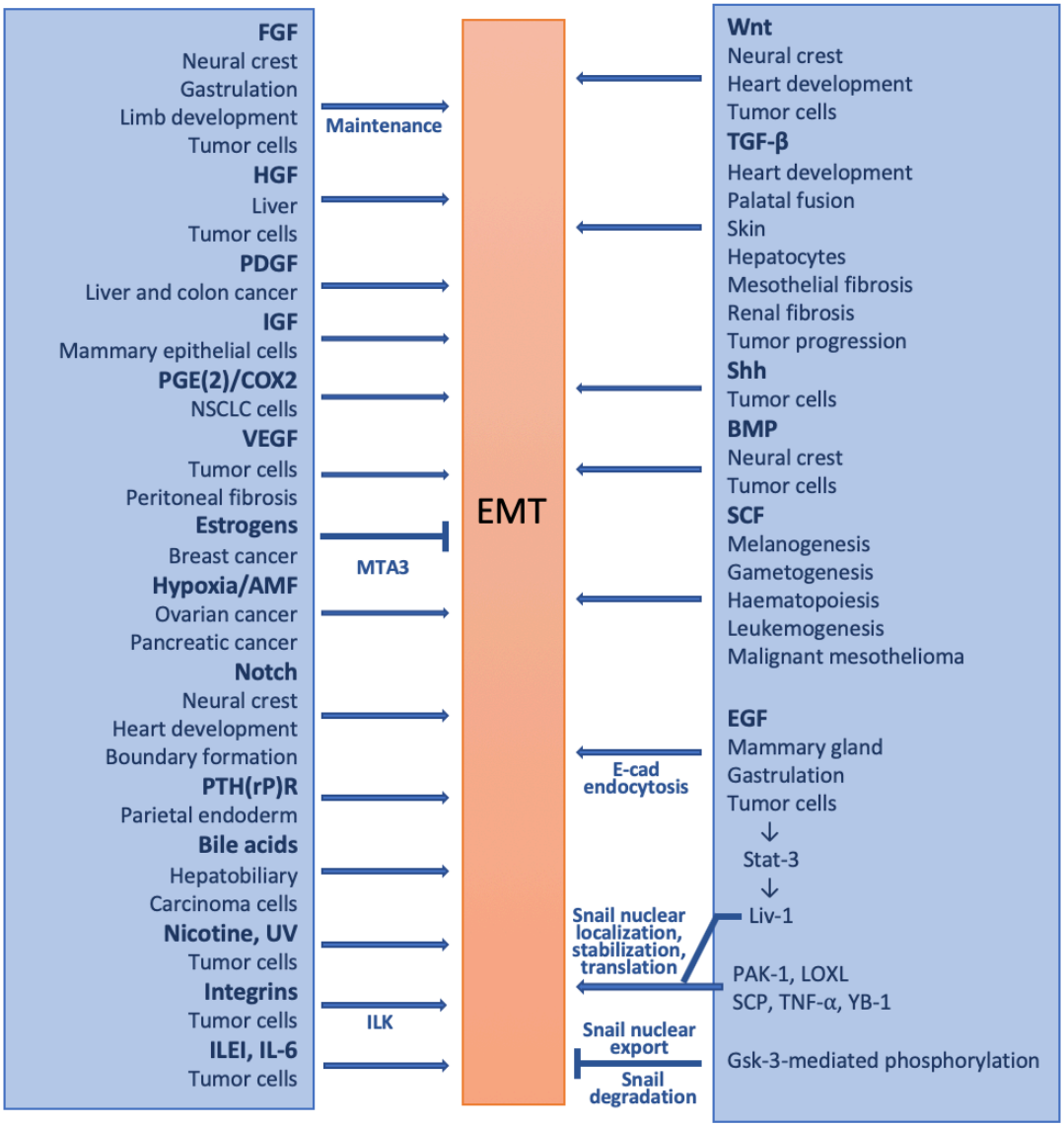


Figure 1-4 EMT Signalling Pathways.

Various signalling and proteins prompt EMT in cells in embryogenesis, organ genesis, and also in human diseases. The above indicates the affected tissues and biological changes after the consequent reactions that promote EMT. Adapted from (Thiery et al., 2009)

Recent studies have indicated that microRNAs can also regulate EMT by controlling EMT inducers (Korpal, Lee, Hu, & Kang, 2008).

The miRNA-200 family, including miR-200a, miR-200b, miR-200c, miR-141 and miR-429 and miR-205 regulate the E-cadherin repressor ZEB1, (closely related to EMT and tumour metastasis), inhibit E-cadherin expression and induce EMT. In contrast, overexpression of these miRNAs reverses EMT and induces mesenchymal transition

(MET). ZEB1 binds to the promoters of miR-200c and miR-141 to inhibit their expression, thereby helping ZEB1 to maintain the phenotype of the stromal cells.

1.2.2 *EMT in disease*

EMT plays an important role in epithelial injury, resulting in organ fibrosis and failure, and has been proven in kidney, respiratory, and hepatic fibrotic process models (Zavadil, Haley, Kalluri, Muthuswamy, & Thompson, 2008).

Fibrosis is due to the pathological activation of fibroblasts. Fibroblasts accumulate and secrete too much collagen. The collagen fibers are stored, and they form a fibrosis collagen network, thus disrupting organ function and causing organ degeneration (Kendall & Feghali-Bostwick, 2014). Research shows that most of these fibroblasts are formed by EMT (Kalluri, 2009; Zeisberg & Duffield, 2010).

EMT is currently considered a major factor in renal fibrosis, which leads to the proliferation of renal interstitial fibroblasts, eventually leading to tubular fibrosis. During renal fibrosis, BMP-7 and TGF-β are two major regulators, and high levels of TGF-β are found in the patient's fibrotic tissue. (Figure 1-5)

Despite the fact that EMT is essential for embryogenesis, organ generation and subsequent for wound healing and organ regeneration, the irregular induction of EMT can be harmful during adult life (Kalluri & Weinberg, 2009). The protein inducing EMT is locked in adult organs, however it can be restarted when the epithelial status of cells is changed, for example, in fibrogenesis (Iwano et al., 2002). SNAI1, one of the EMT promotors, is triggered probably following the TGF-β secretion in kidney fibrosis mouse models (Sato, Muragaki, Saika, Roberts, & Ooshima, 2003). Remarkably, Snail activation disrupts tissue homeostasis and is abundant in prompting EMT, fibrosis, and kidney failure in adult transgenic mice and is greatly actuated in renal fibrosis caused by nephrectomy (Boutet et al., 2006).

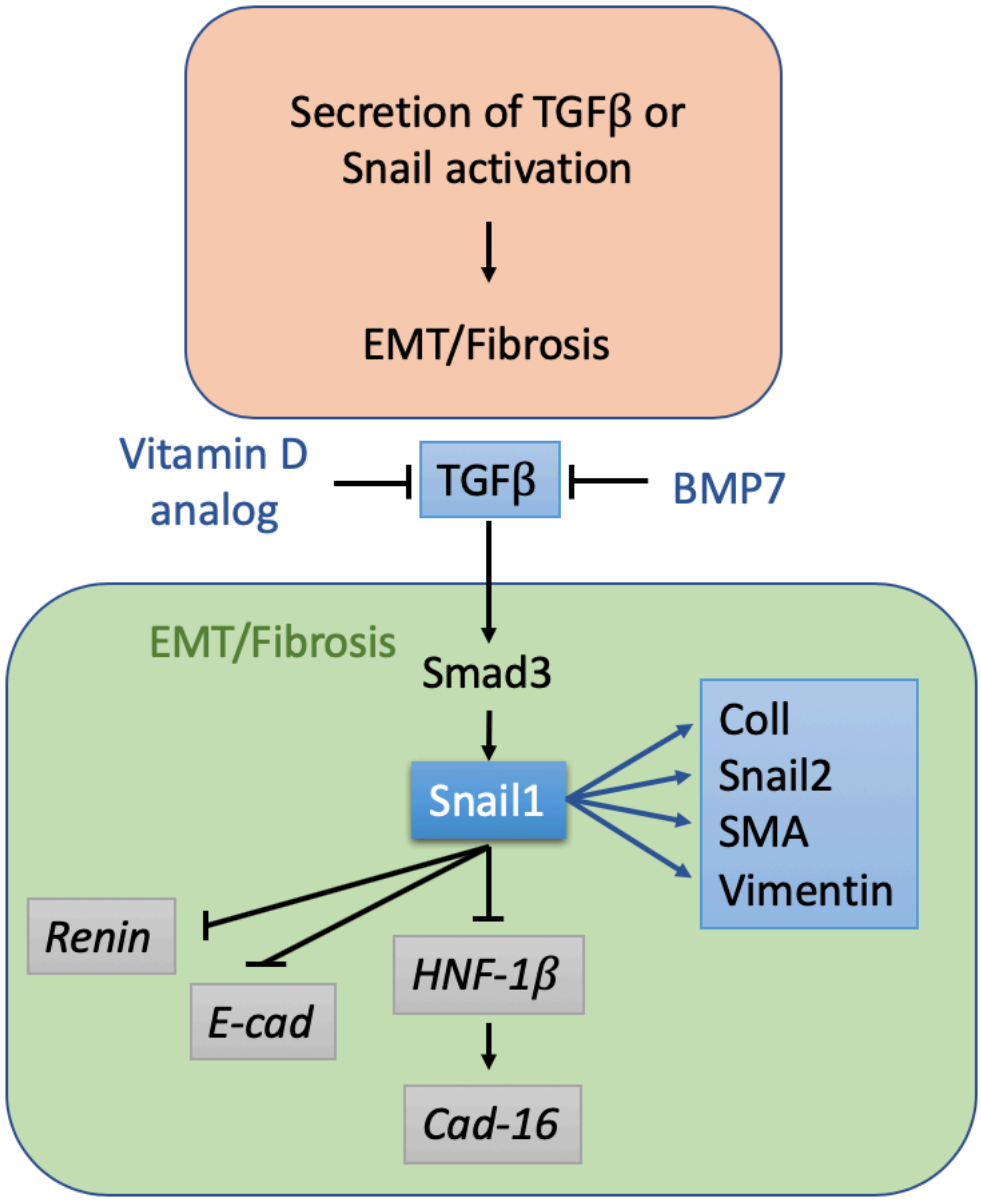


Figure 1-5 TGF-β signalling regulates several pathways

TGF-β signalling controls numerous pathways including promoting SNAI1 stimulation, that initiates EMT and helps epithelial cells transform into myofibroblasts which produce and secret collagen I. BMP-7 or vitamin D analogue may suppress TGF-β signalling which potentially decreases fibrosis in the kidneys. The bold characters represent pathways triggered by TGF-β signalling.(Thiery et al., 2009)

1.2.3 *EMT in tumour progression*

A number of studies have confirmed that EMT is vital in tumour invasion and metastasis, and the invasiveness of tumour cells is significantly enhanced after EMT (Figure 1-6).

Recent studies have confirmed that EMT is present in a number of tumours, like colon, breast, prostate cancer, liver cancer, lung cancer and cervical cancer (Brabletz, Kalluri, Nieto, & Weinberg, 2018; Tania, Khan, & Fu, 2014).

EMT enables the invasion required in the initiation of tumour metastasis. Cell-cell adhesion mediated by repression of E-cadherin of cancer cells in the primary tumour is lost, while adhesion of basement membrane is lost via its basal surface increasing invasion and consequently joining the circulation. After that, cells leave the circulation to undertake the reverse mesenchymal-epithelial transitions (MET) to settle down at the metastatic location. Therefore, the invasion and then metastasis is induced and accomplished by EMT and MET (Chaffer & Weinberg, 2011). Furthermore, EMT gives cells mesenchymal features, as well as enables them to gain several other properties like drug resistance and evasion of apoptosis.

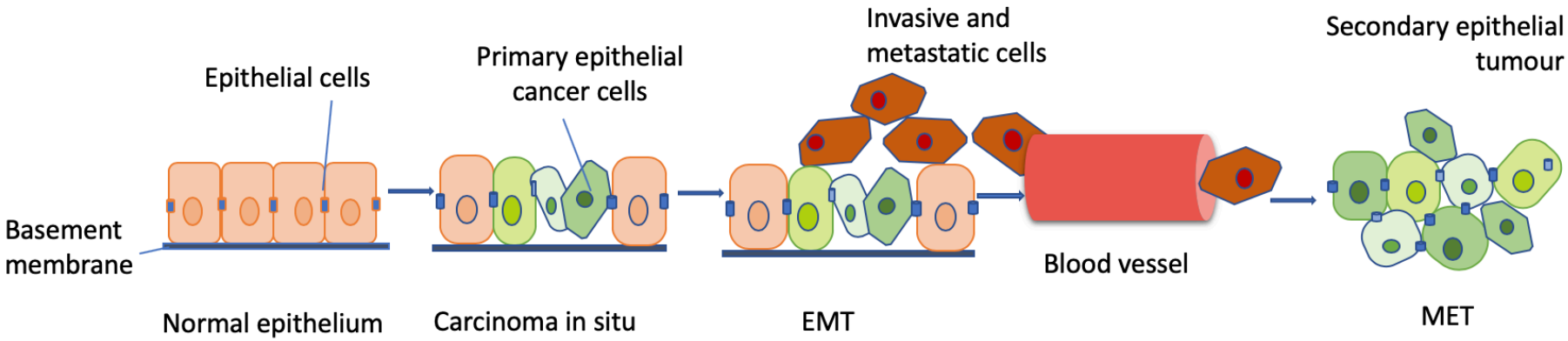


Figure 1-6 EMT plays a role in tumour progress.

There are some phases undergoing the initiation of tumour progression, invasion and metastasis. Normal epithelial cells lose their polarization and adhesion to become invasive. Then the invasive and metastatic cells leave the tumour site to go into the blood vessel. EMTs also involve the next step: tumour cells leave the circulation at a distance to form metastases, which potentially undergo METs and therefore reverse to epithelial cells. (Kalluri & Weinberg, 2009)

1.2.4 *EMT as a cancer therapeutic target*

There is growing evidence that EMT and MET transitions play a crucial role in the regulation of cellular plasticity. At the same time, they may also become important targets for the treatment of tumours, metastasis and tumour tolerance. The variety of clinical results brought by EMT has shown that EMT is significant; therefore, inhibition of EMT has become a very promising treatment. However, the complexity of the signal pathways that regulate the EMT process, combined with the existence of a MET conversion mechanism that reverses the results of an EMT, makes the situation even more complicated. In addition, it is not known which tumour cells should be treated and at which stage of the tumour development. Recent studies have shown that tumour cells are already starting systemic dissemination at an early stage of tumour development. It is too late to give EMT suppression treatment after the tumour is clinically diagnosed. However, if tumour metastasis is a continuum (i.e., one metastasis spreads to the next), the use of EMT suppression therapy should prevent subsequent tumour metastases.

The TGF-β signal is one of the EMT-inducing signals and is now the most clearly studied. Interestingly, while TGF-β inhibits the growth and proliferation of normal epithelial cells, it promotes tumour invasion and metastasis, tumour neovascularization, and suppresses anti-tumour immune responses in the tumour microenvironment at the late stage of cancer. Interestingly, TGF-β signalling can be pro-tumorigenic or tumour suppressive. Recently, David et al. published an article on TGF-β promoting pancreatic cancer cells entering lethal EMT transformation to suppress tumours. In TGF-β-sensitive pancreatic ductal adenocarcinoma (PDA) cells (EMT could be induced by TGF-β in PDA cells), the switch of TGF-β-induced Sox4 from a tumorigenesis enhancer into a cell death inducer makes EMT fatal. The mechanism is supposed to be relating with remodelling of the TF signalling with EMT, involving the suppression of the Klf5 which collaborates with Sox4 in tumorigenesis and inhibits cell death stimulated by Sox4 (David et al., 2016).

Despite the current advances in EMT understanding, we have yet to develop a complete EMT-targeted cancer drug. Inhibition of the epidermal growth factor receptor (EGFR) is a significant treatment for non-small cell lung cancer (NSCLC) patients. In both

prognosing NSCLC and valuing treatment efficiency, EMT monitoring has been developed as a probe as expression of mesenchymal markers correlates with a bad prognosis and a poorer response to EGFR-TKIs. The EMT process is also linked to EGFR-TKI resistance in NSCLC. (Jakobsen, Demuth, Sorensen, & Nielsen, 2016) There are lots of signalling pathways and proteins that induce and modulate EMT, including NFκB pathway, Wnt and Notch proteins, TGF-β, and RTKs and activating GFs such as IGF1, GAS6, HGF, FGF and EGF. In the occurrence of EMT triggered by Notch, EGFR-TKI treatment is the inducer as EGFR signalling generally suppresses the Notch pathway, which results in EMT initiation (Arasada, Amann, Rahman, Huppert, & Carbone, 2014). New drugs that target tyrosine kinase activity directed against different receptors in the EMT process, including vascular endothelial growth factor receptor EGFR, insulin-like growth factor 1 receptor IGF1R, PDGFR, FGFR, etc. are still in phase I clinical trials or in preclinical testing. The main problem in this type of study is the lack of a specific atraumatic marker of tumour metastasis during treatment that causes minimal tissue injury. We believe that the research on EMT and tumour metastasis will open up new horizons for the treatment of malignant tumours.

It is unclear which signalling pathways to suppress during the EMT process to achieve the best possible outcome while minimizing side effects. The similarity between the EMT program and the normal stem cell program makes the toxic side effects of EMT inhibition therapy a major stumbling block. At the same time, the reversible nature of the EMT program and the MET program casts doubt on the efficacy of EMT suppression therapy. Another key issue is that tumour metastasis often evolves over years, or even decades, such as breast cancer - so how can we determine whether EMT suppression is effective before the tumour metastasis?

The problems above indicate that although we have gone through a great deal of effort, discovered some of the molecular mechanisms underlying EMT and MET and their importance for clinical oncology patients, and developed effective anti-cancer therapy based on EMT and MET mechanisms, there are still some important basic issues to be solved. However, we believe that with the unrelenting efforts of the researchers, these problems will surely be resolved one by one in the near future.

Also, as one of the important phenomena in the process of tumorigeneses, EMT is an important mechanism in tumour cells invasion and migration and secondary metastasis. At present, the research on EMT is gradually deepening, and its role in cancer is being continuously uncovered, and the molecular mechanism of EMT continuously revealed. With the deepening of research, some new fields such as the interaction of EMT and the tumour matrix, the relationship between EMT and tumour angiogenesis, between the key events blocking EMT and the suppression of tumour invasion and metastasis will also be revealed. This will also provide new ideas and methods for cancer therapy.

1.3 Alternative Splicing

Alternative splicing is a very important gene regulation mechanism. It refers to the process of producing different mRNA splicing isoforms from one mRNA precursor by differential splicing (selecting different splice site combinations), then producing different proteins with different or mutually antagonistic functions and structural features, or different phenotypes due to differences in expression levels in the same cells (Chow, Gelinas, Broker, & Roberts, 1977; Y.-J. Kim & Kim, 2012; Konarska, Grabowski, Padgett, & Sharp, 1985; Pajares et al., 2007) (Figure 1-7). It is essential in differentiation, development, normal physiology, abnormal pathologies even involving cancer, and it contributes to the variation of protein in advanced eukaryotes.

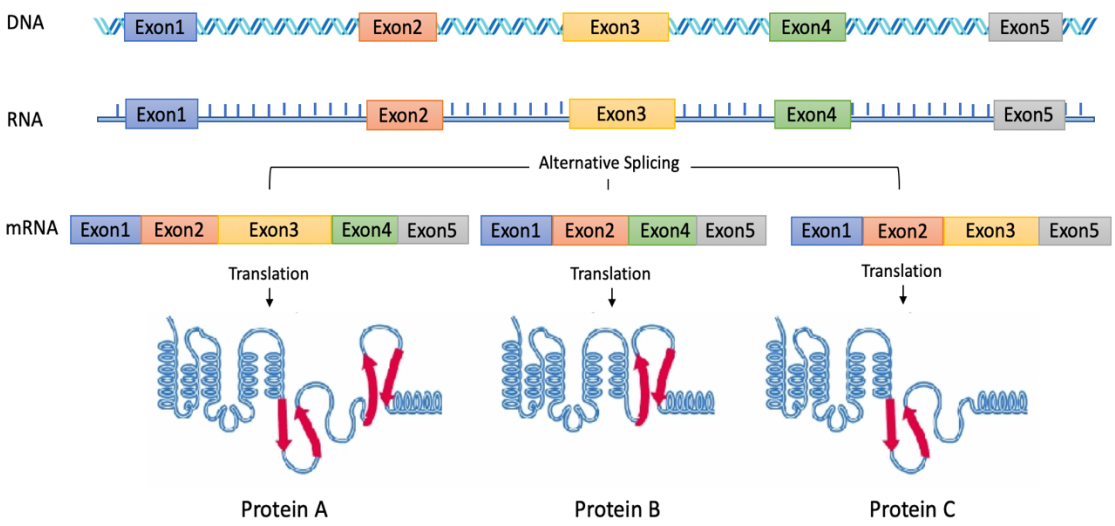


Figure 1-7 An outline example of alternative splicing.

Alternative splicing involves the process of producing several mRNA transcripts from one certain gene. Adapted from (Chow, Gelinas, et al., 1977)

During splicing, a large protein-RNA complex forms in the cell that becomes a spliceosome. Spliceosomes recognize exons and remove introns. Spliceosomes consist of more than 120 proteins and 5 small nuclear RNAs.

Spliceosome splicing involves two steps of biochemical reactions, both of them require trans-esterification reactions between the RNAs (Shi, 2017). Spliceosome and self-splicing esterification reactions occur in a specific order. First, at branchpoint A of an intron, the nucleotide will undergo transesterification with the first nucleotide of this intron to form two RNA molecules, one being an intron lariat, and the other being the exon before intron. Then, the last nucleotide of the first exon will undergo transesterification with the first nucleotide of the second exon, ligating the exon and releasing the intron lariat (McManus & Graveley, 2011; Shi, 2017) (Figure 1-8).

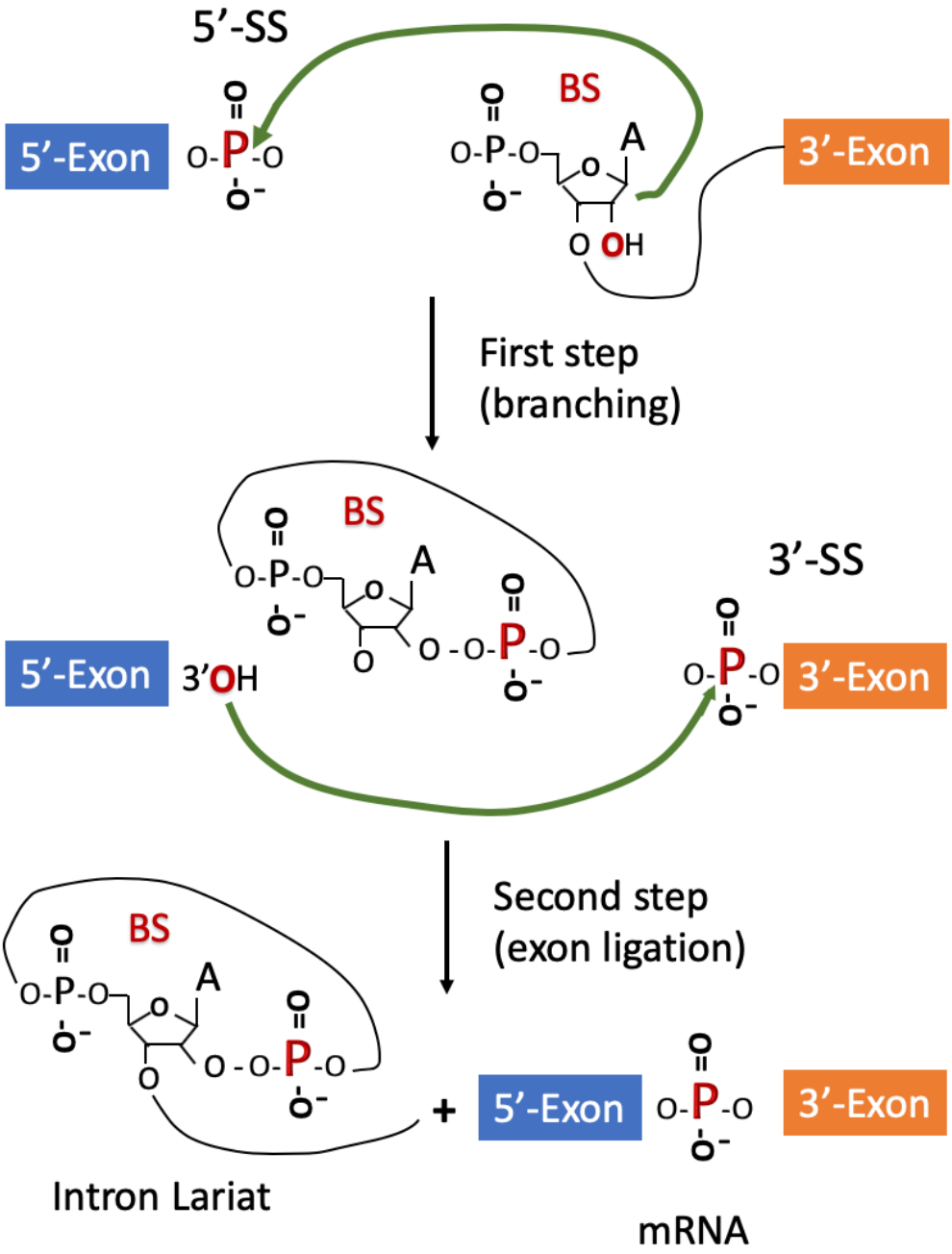


Figure 1-8 Two transesterification reactions are involved in spliceosome splicing: branching and exon ligation.

First, the 3’-OH of 3’-intron and the 5’-end phosphate of the 5’-intron were connected to free the 5’-exon and a lariat was produced intermediately. Second, the two exons are ligated by a combination of 3’-OH of the 5’-end phosphate of the two exons and then the intron lariat was released. Adapted from (Shi, 2017)

1.3.1 *Pre-mRNA Splicing*

In 1958, the "sequence hypothesis" was first proposed by Crick. In this hypothesis, the codon encoding the amino acid sequence of the protein is offered by the nucleotide of the genetic material, which is the foundation of the "central rule of molecular biology" - the double-stranded DNA is transcribed into a single-stranded RNA molecule, which is then translated into a protein (Crick, 1958). Scientists first thought that mRNA molecules were translated by ribosomes through copying DNA sequences exactly. Roberts and Sharp then proposed the "split gene" hypothesis through their study of viral genes (Chow, Roberts, Lewis, & Broker, 1977). They found that in viruses, some mRNAs are shorter than the DNA they transcribe, and have large interference gaps, called introns. Soon most eukaryotic genes were also found to have this molecular structure, and the coding sequence (exons) was interrupted by non-coding sequences (introns). During transcription, the introns of Pre-mRNA are removed, and the exons are relegated by the splicing process to produce mature mRNA transcripts (Figure 1-9).

It is now commonly believed that the theory that one gene holds the message for creating one amino acid polypeptide chain (Beadle & Tatum, 1941) has proven to be over-simplified. In fact, there are much fewer genes in the human genome than expected, particularly when compared to the number of proteins in the human proteome.

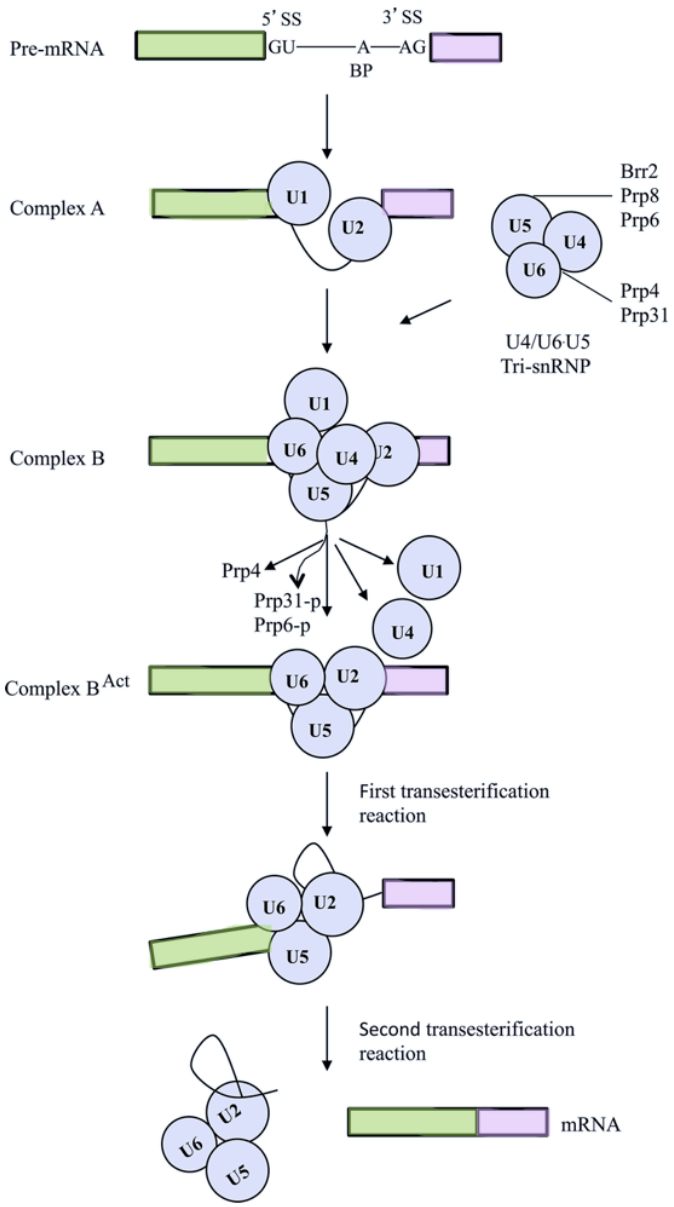


Figure 1-9: snRNPs in Pre-mRNA splicing.

Pre-mRNA is transcribed from a gene, introns removed, and exons re-joined by the process of splicing to create mature mRNA transcripts under the function of small nuclear ribonucleoproteins (snRNPs). Base pairing of U1 to 5'ss and recognition of BP by U2 (formation of complex A) are followed by the integration of preformed U4/U6-U5 tri-snRNP to form complex B. Prp4, Prp31, and Brr2, Prp8, Prp6 are components of U4/U6 snRNP and U5 snRNP, separately. Then Complex B is activated by the phosphorylation of Prp6 and Prp31 (complex B^{Act}). U1, U4, Prp4, Prp6, and Prp31 are released from the activated spliceosome that catalyzes the two transesterifications steps for intron excluding. Adapted from (X. L. Gao et al., 2016)

1.3.2 *Alternative splicing: a mechanism to increase proteome diversity*

It is predicted that there may be about 19,000 genes in the human genome, about 14,000 in fruit flies and about 19,000 in *C. elegans* (Ezkurdia et al., 2014). There seems to be a significant difference between the biological complexity and the number of genomic genes. The reason is in the proteome. Mechanisms such as gene rearrangement, RNA editing, and alternative splicing can produce numerous proteins from a single gene, allowing the number of proteins in the proteome to exceed the number of genes in the genome. Among them, variable splicing is the most important mechanism for expanding protein diversity regarding the number of genes affected and the range of biological species (Adams et al., 2000; Consortium, 1998; Ewing & Green, 2000; Pennisi, 2000).

There are several important questions about alternative splicing - firstly, the frequency of alternative splicing. In 1977 Walter Gilbert put forward the concept of alternative splicing (Maxam & Gilbert, 1977). In 1980, Baltimore discovered the first alternative splicing in mouse IgM gene to generate membrane-type and secretory IgM (Alt et al., 1980). By 2001, using classical molecular biology experiments, a total of only a few hundred genes with AS were discovered and it was speculated that around 5% of genes in higher eukaryotic organisms have alternative splicing (Graveley, 2001; Modrek & Lee, 2002).

High-throughput genomic sequencing and expressed sequence tags (EST) sequencing make it possible for bioinformatics methods to study alternative splicing. ESTs are derived from fully processed mRNAs, which provide a broad range of mRNA diversity samples. This diversity can be analysed by computer. In the last few years, several research groups have analysed data from human genome sequencing project and found consistently that about 35% -60% of human genes have alternative splicing patterns. Moreover, for most genes, only a few ESTs or even no ESTs are reported for each gene; ESTs are not full-length mRNAs, but mostly at the 5 'and 3' ends of the mRNA; ESTs are derived from limited tissue at a limited development stage; it is highly likely that more alternative splice variants are not shown in the current EST library. Therefore, the actual frequency of alternative splicing may be higher than predicted.

More and more human genes have been revealed to undertake alternative splicing (Modrek & Lee, 2002; Pan, Shai, Lee, Frey, & Blencowe, 2008; E. T. Wang et al., 2008). New data using RNA sequencing has shown that about 95% of human multi-exon genes have an alternative splicing process (Pan et al., 2008), and according to the latest human genome annotation information (GENCODE Human Annotation, the data provided by Version 28, http://www.gencodegenes.org), 58381 genes (including 19,901 protein-coding genes) in the human genome produce a total of 203,835 transcripts (including 82335 protein coding transcripts). The number of transcripts encoded on average by one gene reached 4.14, which indicates the great contribution of alternative splicing to protein diversity.

Secondly, another issue is the diversity of single gene alternative splicing (Graveley, 2001). A single gene can produce multiple transcripts in several ways, such as different transcription initiation sites, alternative splicing, selection of different tailed signal sites, and RNA editing. There are seven modes of alternative splicing: exon skipping; alternative 3′-SS; alternative 5′-SS; mutually exclusive exons; intron retention; usage of alternative promoters and alternative polyadenylation. The effect of alternative splicing on the structure of proteins is also diverse, such as the increase or decrease of one to several hundred amino acids in the polypeptide chain; the presence or absence of a domain; ineffective translation if alternative splicing changes the reading frame, mRNA degraded by the monitoring system - the nonsense-mediated mRNA decay (NMD).

The production of 10-12 isoforms from a single gene through alternative splicing is quite common. Some genes even produce thousands of splicing isoforms. The most prominent example is the Dscam gene of Drosophila melanogaster, which produces over 38,000 mRNA isoforms through alternative splicing. The Dscam gene encodes a neuronal axon-targeting receptor, which has an extracellular domain consisting of 10 immunoglobulin repeats. Fortynine different splicing isoforms were found by random sequencing of 50 cDNA clones of the Dscam gene, indicating that there are at least a thousand species of splicing isoforms even if they are not theoretically possible. Genes such as Neurexins, N-Cadherins and calcium-activated potassium channels in humans also have similar highly diverse splicing isoforms (Schreiner, Simicevic, Ahrne, Schmidt, & Scheiffele, 2015; L. J. Tian et al., 2001).

1.3.3 *Modes of alternative splicing*

Seven basic modes of alternative splicing are recognized (Figure 1-10) (Di et al., 2019):

1) Exon exclusion: a whole exon is skipped or cassette;
2) Mutually exclusive exons: one of the two exons is included and the other is excluded in splicing;
3) Intron retention: an intron sequence is retained by mis-splicing;
4) 5' Alternative donor site: a 5' alternative splice site competes with the natural ones to the loss of part of an exon or to include part of an intron in the mRNA;
5) 5' Alternative donor site: a 3' alternative splice site competes with the natural ones to the loss of part of an exon or to include part of an intron in the mRNA;
6) usage of alternative promoters: use of alternative promoter results in exon skipping;
7) alternative polyadenylation: use of poly A results in following exons exclusion.

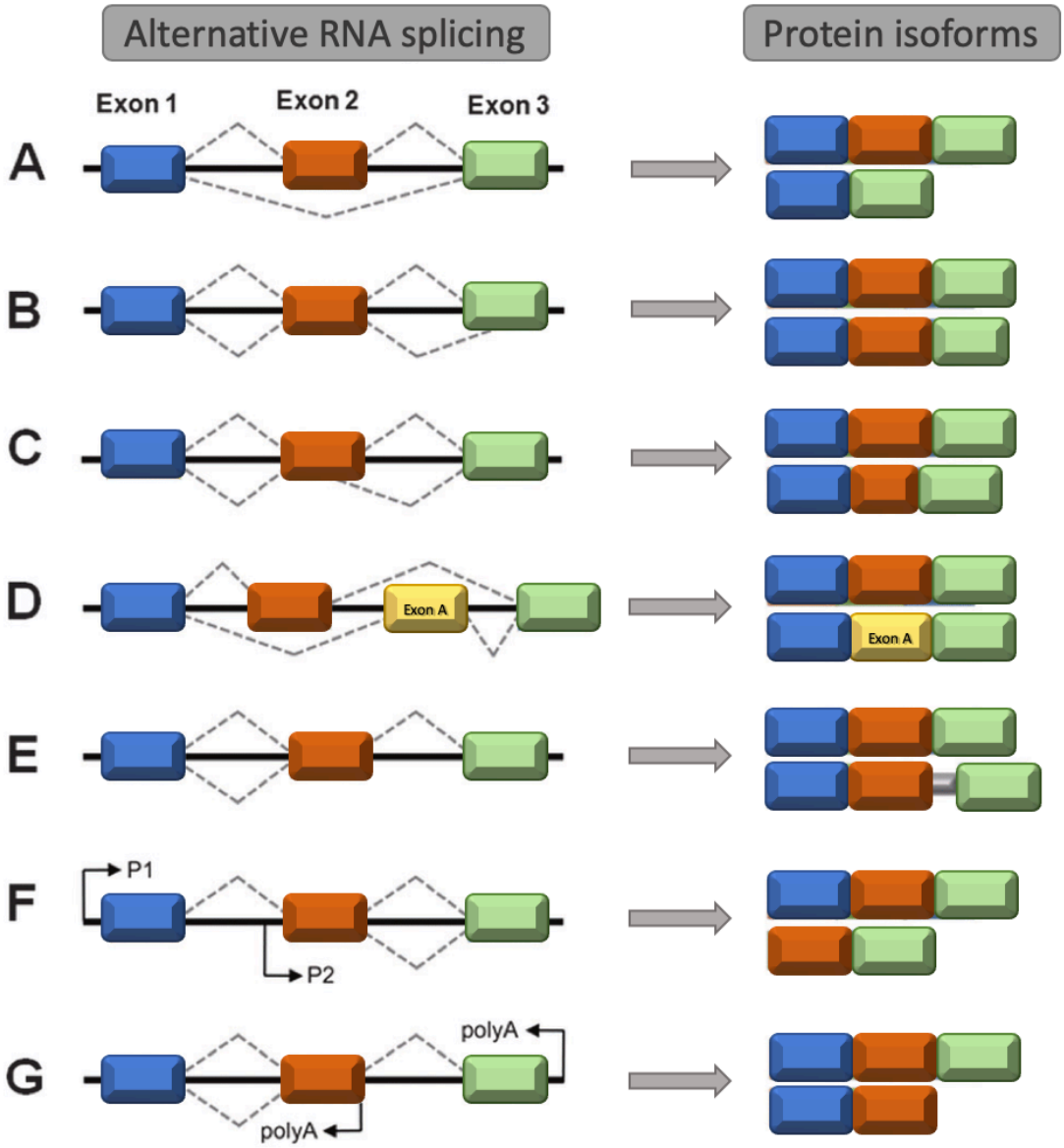


Figure 1-10 Different patterns of alternative splicing

Common types of alternative splicing are shown as above: ***A****: exon exclusion – skipping introns and entire exon 2;* ***B:*** *alternative splice sites – exon 3 has a 5' alternative splice site.* ***C:*** *alternative splice sites – exon 2 has a 3' alternative splice site. The two sites compete with the entire ones which result in a part exon or intron inclusion* ***D:*** *mutually exclusive exons – exon 2 or exon A is excluded, only one is included;* ***E:*** *intron retention – including intron 2;* ***F:*** *usage of alternative promoters – in use of P2 exon 1 is excluded* ***G:*** *alternative polyadenylation – exon 3 is excluded by use of poly A of exon 2. P: promoter, poly A: site of polyadenylation. Adapted from (Di et al., 2019).*

1.3.4 *Regulation of alternative splicing*

In alternative splicing regulation, splice factors, splicing related proteins and RNA, as well as splicing enhancers and silencers play an important role (Figure 1-11).

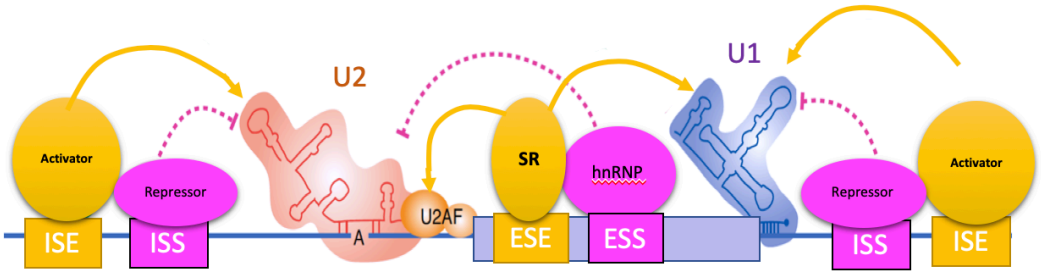


Figure 1-11 Several regulatory proteins and sequences modulating alternative splicing.

There are four categories of sequences involved in regulating alternative splicing: intronic splicing enhancers (ISEs), intronic splicing silencers (ISSs), exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs). Adapted from (McManus & Graveley, 2011).

Splicing enhancers and silencers include intronic splicing enhancers (ISEs), intronic splicing silencers (ISSs), exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs). (McManus & Graveley, 2011) The enhancers are identified by activating proteins that often come from the SR protein family. Silencers combine with suppressors that are most often from the hnRNP protein family. Activators generally help spliceosomal components combine to the regulated splice site while suppressors likely prevent the spliceosomal components from combining or functioning, which is due to the binding sites. In splicing, the combination of ESE/ISE and serine/arginine-rich (SR) protein could help with binding U1 small nuclear ribonucleic particles (snRNP) and U2 snRNP to the splice site to facilitate the splicing and exon inclusion. However, the binding of exon splicing silencer ESS/ ISS and heterogeneous nuclear ribonucleoprotein (hnRNP)

prevents U1 snRNP and U2 snRNP binding to the splice site, impedes the splicing and thus excludes exons from mature mRNA (exon exclusion).

Splice factors play an auxiliary role in splicing by binding to ESE/ESS. The fact that most ESEs are located adjacent to the splice site they regulate helps to recruit splicing factors to the splicing site; changing their location will alter splicing activity or even cause them to become trans-acting factors.

During splicing, some proteins are required to recognize enhancers or silencers in exons and introns that, when combined with enhancers or silencers, can recruit U1 snRNP, U2 snRNP, or serine/arginine-rich splicing factor 1 (SRSF1) to constitute a spliceosome. Classically ESE binds to the SR protein, whereas the ESS binds to the hnRNPs. Generally, SR protein can promote exon inclusion, while hnRNPs prevent exon inclusion; though, in some genes it works differently, such as an exon of GTPase *Rac1* being blocked by the SRSF1, whereas an abnormal splicing of ceramide blocks exon inclusion via the SR protein (Goncalves, Matos, & Jordan, 2009; Sumanasekera et al., 2012).

Polypyrimidine tract binding protein (PTB) is a well-studied hnRNP. PTB competes with SRp30c for regulatory elements of the hnRNPA1 gene (Paradis et al., 2007). In addition, there is competition between SR proteins such as SRp20 and SRSF1 in the *Rac1b* gene (Goncalves et al., 2009). Splicing regulatory proteins not only act as antagonists to each other but also as collaborators with each other, for example FOX-3 interacts with PSF and promotes splicing in neurons (K. K. Kim, Kim, Adelstein, & Kawamoto, 2011). Splice factor expression is one of the important level regulating splicing, and splice factors usually also have the ability to self-regulate. Expression levels of splice factors by self-regulating are also generated by splicing isoforms, such as FOX, SRSF1 (Damianov & Black, 2010; Sun, Zhang, Sinha, Karni, & Krainer, 2010).

AS in the non-coding areas of the mRNA may disturb the translation and mRNA stability by nonsense-mediated mRNA decay (NMD) depending on the translation machinery (Maquat & Carmichael, 2001).

1.3.5 *Splicing in disease*

Over the last few years, the correlation between alternative splicing and human diseases has been gradually revealed. Changes in the ratio of splicing isoforms of some key proteins play a role in the development and progression of pathogenic processes.

It is revealed that the genetic disorders were caused by splicing mutations; however, the mutations might be considered less frequent than in reality (Anna & Monika, 2018). Research demonstrated that 15% of human genetic diseases were caused by splicing mutations, ranging from neurological to myogenic and metabolic disorders (Faustino & Cooper, 2003; Krawczak, Reiss, & Cooper, 1992; Sterne-Weiler & Sanford, 2014). Synchronous mutations in disease do not cause changes in the encoded protein sequence but may alter ESE and ESS on exons or introns, thereby affecting the splicing process. Similarly, since some mutations cause RNA terminators to appear prematurely, not only does this RNA have no function in the encoded protein, but it may directly affect the splicing process of other normal RNAs and cause disease.

The classic examples of mutations in *cis*-acting elements that trigger splicing defects are the Tau protein disorders represented by frontotemporal dementia and Parkinsonism (FTDP), muscular dystrophy (MD), and spinal muscular atrophy (SMA) (Licatalosi & Darnell, 2006; Spillantini, 2003).

Mutations that occur in the trans-acting element genes regulate the proteins that control the splicing process and cause disease. *SF3B1* and *U2AF1* are the genes respectively encoding U2 snRNP and U2AF1 (both are protein complexes involved in splicing), and the mutations in these two genes are tumour-associated (Papaemmanuil et al., 2011).

1.3.6 *Cancer and alternative splicing*

The biological origin, progression, and metastasis of cancer are related to the diversity of mRNA. Cancer-specific alternative splicing is a ubiquitous phenomenon and a major post-transcriptional regulatory mechanism involving many cancer types. Every 'hallmark of cancer' is connected to a splicing change, promoting the tumour to be more aggressive (S. Oltean & Bates, 2014). In tumourigenesis and metastasis, genomic steadiness is disturbed by oncogenic and cancer suppressing proteins encoded by AS, e.g. LEF1, TP63, HNF4A, RASSF1 and BCL2L1 (Carstens, Eaton, Krigman, Walther, &

Garcia-Blanco, 1997; Davuluri, Suzuki, Sugano, Plass, & Huang, 2008; E. Kim, Goren, & Ast, 2008; Pajares et al., 2007; Rajan, Elliott, Robson, & Leung, 2009; Venables, 2006). (Figure 1-12)

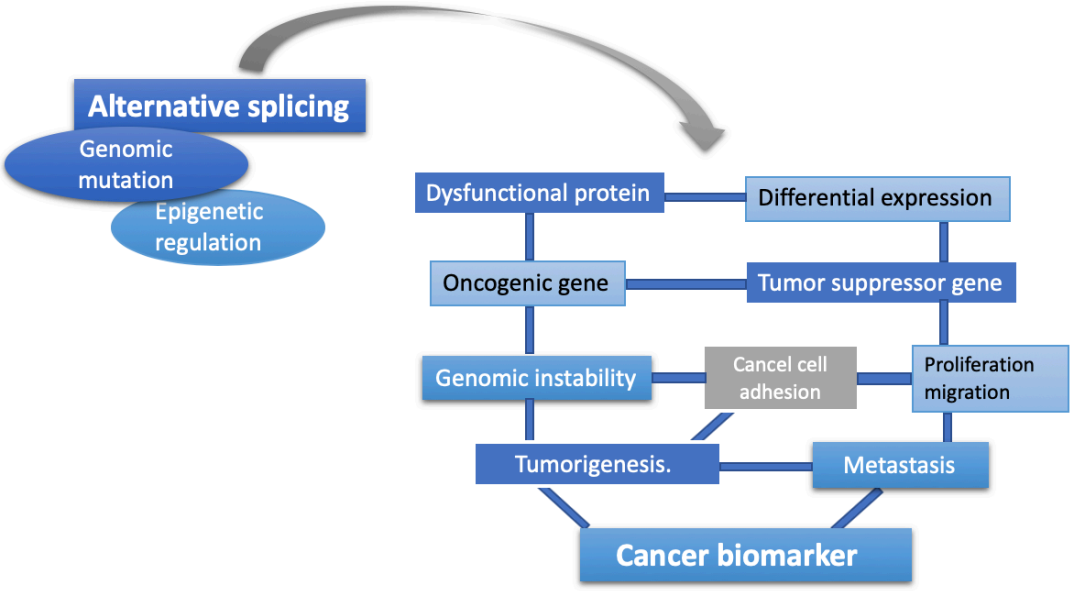


Figure 1-12 Alternative splicing functions in tumour progression

The formation of the tumour is induced by the abnormal proteins in tumour cells. The different isoforms of oncogenic and tumour suppressor genes in tumours compared to normal tissues might be prospective markers to distinguish normal and tumour cells. Adapted from (Y.-J. Kim & Kim, 2012).

While it is still not fully revealed how AS functions in tumours, several of the vital stages of the typical splicing pattern are perceived in tumours (Miura, Fujibuchi, & Sasaki, 2011).

Alternative splicing is an essential regulatory step in all of the cancer hallmarks, e.g. apoptosis and proliferation. RNA Binding Motif Protein 4 (RBM4) is a splicing factor capable of binding to intron splicing facilitating elements to regulate splicing. RBM4 switches splicing of Bcl-x to control the balance between pro- and anti-apoptotic pathways and, thus, promoting tumour cell death and inhibiting cancer progression. More importantly, through high-throughput sequencing it was found that RBM4 can also regulate the expression of another very important splicing factor, SRSF1, which is

known to encourage cell proliferation and decrease apoptosis, and further affect the activation of the downstream mTOR signalling pathway which can promote tumour development through proliferation. (Y. Wang et al., 2014)

Angiogenesis, the forming of new blood vessels, is key to tumour development and regulated by AS. Vascular endothelial growth factor A (VEGF, also referred to as VEGF-A) which is controlled by several splicing factors, is a key inducer of vascular formation in cancer (Biselli-Chicote, Oliveira, Pavarino, & Goloni-Bertollo, 2012). VEGF has multiple alternative splice sites, which increases the diversity of VEGF-A isoforms. AS of exon 8 of VEGF-A is the vital factor converting pro-angiogenic VEGF-Axxx isoform to anti-angiogenic VEGF-Axxxb isoform (Harper & Bates, 2008). For example, VEGF-A_{165} excites immigration and cell growth, promotes vasodilatation, improves permeability in endothelial cell *in vitro*, constantly boosting formation, growth and permeability of blood vessels, and pathological retinal neovascularization *in vivo*, while VEGF-A_{165b} has the opposite functions, and reduces some responses in tumours induced by VEGF-A_{165} (Bates et al., 2002; Bates & Curry, 1996; Bevan et al., 2008; Ferrara, Houck, Jakeman, Winer, & Leung, 1991; Harper & Bates, 2008; Ku, Zaleski, Liu, & Brock, 1993; Mitchell et al., 2006).

For another hallmark of cancer – invasion and metastasis, alternative splicing also plays a vital regulation role. During cancer progression, epithelial-mesenchymal transition (EMT) is an essential step helping tumour cells to gain more invasive and aggressive phenotypes. The EMT process involves a set of biological changes in transcription, post - transcriptional modifications and alternative splicing. One of the well-known genes alternatively spliced in EMT is fibroblast growth factor receptor II (FGFR2). FGFR2 has two mutually exclusive alternative exons - exon IIIb and IIIc. Exon IIIb is selected in epithelial cells while in EMT, exon IIIc is selected to produce a mesenchymal phenotype. Besides the switch between the two exons, there are a series of functional changes inside the cells. Researchers found that the RNA binding proteins hnRNPA1 and PTB, which had high expressing level in tumours, played a role in exon IIIc exclusion (Yasumoto, Matsubara, Mutaguchi, Usui, & McKeehan, 2004). Two splicing regulatory proteins, epithelial splicing regulatory protein 1 and 2 (ESRP1 and ESRP2), were recognized by exploring genes that were involved in helping IIIb inclusion. ESRPs are

expressed specifically in epithelial cells while lost in EMT. Exon IIIc exclusion of *FGFR2* in mesenchymal cells could be induced by overexpressing ESRPs.

In addition, AS contributes to protecting tumours from the drug treatment – drug resistance. Taking *BRAF* (a human gene that encodes a protein called B-Raf), an oncogene, as an example, it has been found that cancer cell lines with Vemurafenib (a kind of targeted cancer drug prohibiting cells expressing BRAF protein from stimulating some tumour cells) resistance produce a truncated BRAF variant protein, directly affecting cell growth and avoiding the drug effect. Generating of this variant is due to abnormal AS – an absence of a part of exon. (Pratilas, Xing, & Solit, 2012)

BRCA1 splicing and poly ADP ribose polymerase (PARP) inhibitor in breast cancer therapy is another example of resistance to therapy. PARP1 is critical for repairing damaged DNA and is a vital protein which PARP inhibitors take effect on. PARP inhibitors are particularly useful in patients deficient in DNA repair due to mutations in the *BRCA1* gene. But unfortunately, like many other treatments, cancer cells eventually develop resistance. The researchers found that specific PARP1 mutations inhibit the combination of proteins and DNA, leading to PARP inhibitors being unable to monitor DNA damage in cancer cells. The mutated *BRCA1* gene can protect tumour cells from dysfunctions of DNA repair of PARP1 and therefore PARP inhibitors. (Pettitt et al., 2018; Siegfried & Karni, 2018)

1.4 Epithelial splicing regulatory proteins

Warzecha and his lab indicate that Epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) are main regulators that switch splicing during EMT and are specific to epithelial phenotype (Warzecha et al., 2010). ESRPs control a large number of genes splicing to the epithelial phenotype during EMT. In EMT, ESRPs are usually lost, therefore their target genes splice differently which result in a different phenotype - mesenchymal phenotype. AS involved in EMT was observed in primary breast cancer biopsy, and the change of ESRP1 expression is an important reason for switching mobile cells to gain epithelial properties (Shapiro et al., 2011; Warzecha, Sato, Nabet, Hogenesch, & Carstens, 2009; Warzecha, Shen, Xing, & Carstens, 2009). Meanwhile,

other research demonstrated that depletion of ESRPs resulted in EMT (Horiguchi et al., 2012).

It is suggested that the ESRPs are one of the most crucial epithelial-specific genes. Mesenchymal transcription factors, e.g. TWIST, SNAI1/2, or ZEB1/2 are main suppressors of ESRPs, either acting direct or subtly to accomplish a whole phenotype switch in EMT (Warzecha et al., 2010). ESRPs expression is also indirectly down-regulated by TGF-β. Comprehensive patterns of alternative splicing were found induced by TGF-β through downregulating ESRPs, which was the result of TGF-β-mediated increasing delta-EF1 family, delta-EF1 and SIP1 transcription factors (Horiguchi et al., 2012).

1.4.1 *ESRPs as RNA binding proteins*

RNA binding proteins (RBPs) play a key role in gene regulation. At present, only a few RNAs can function alone as ribozymes. Most RNAs bind to proteins to form RNA-protein complexes. The regulation of life activities such as synthesis, alternative splicing, modification, transport and translation play a key role, so studying the interaction between RNA and protein is the key to exploring RNA function.(Lunde, Moore, & Varani, 2007)

There are many types of RBPs, accounting for about 6% to 8% of all proteins encoded by the cell. So far, only a few studies on the function of RBP are clear, indicating Argonaute (AGO), human antigen R (HuR, also known as ELVAL1), ARE/poly(U)-binding/degradation factor 1 (AUF1), Tristetraprolin (TTP also known as zinc finger protein 36 homolog [ZFP36]), and CUG triplet repeat, RNA binding protein 2 (CUGBP2). Many of them are interacting with non-coding RNA (ncRNA) to control the intracellular localization of ncRNA, methylation modification, formation of miRNA silencing complex, and alternative splicing. In the current research on ncRNA such as lncRNA, the function of RBP is considered extremely important.

ESRPs were first known as RBM35a and RBM35b, two well-known RBP proteins. In 2009, Carstens Laboratory reported the characterization of epithelial-specific splicing factors following a cDNA screen, which identified splicing factors that switch splicing of FGFR2 to the epithelial isoform - FGFR2 IIIb (Warzecha, Sato, et al., 2009). The two paralogous

genes (*RBM35A* and *RBM35B*), encoding RNA- binding proteins, are the master regulators of exon IIIb retention. Interestingly, they are not only essential to switch FGFR2 to the epithelial phenotype, but also to CD44, ENAH, and CTNND1 splicing towards epithelial. This suggested that RBM35A and RBM35B are controllers of an epithelial cell-type-specific splicing program, which was definitely confirmed in consequent research (Warzecha, Shen, et al., 2009). Due to the huge raised expression in epithelial cells, the RBM35A and RBM35B genes and their encoding protein products were given the names ESRP1 and ESRP2 (Warzecha, Sato, et al., 2009).

1.4.2 *The role of ESRPs in tumour progression*

ESRPs are thought to be tumour suppressors by acting as master regulators of the splicing decisions that define the epithelial phenotype (Newman et al., 2006; Warzecha, Shen, et al., 2009).

It has been shown that ESRPs bind to the *cis*-element in the intron 8 of FGFR; therefore they inhibit the exon IIIc inclusion and trigger the exon IIIb inclusion, which result in expression of epithelial type proteins (Hovhannisyan, Warzecha, & Carstens, 2006). Researchers proposed that ESRPs are important regulators of EMT and essential in keeping epithelial properties and therefore inhibiting tumour transformation and conversion to cells with mesenchymal properties (Dittmar et al., 2012). Previous work on this field also recognised ESRPs as controllers of a complicated alternative splicing regulating system that supplements a significant post-transcriptional level to the modifications in gene expression. This progress results in complicated alterations in cell morphology, polarization, and behaviour that enable transitions between epithelial and mesenchymal cell status and involve EMT during cancer development and fibrosis (Warzecha & Carstens, 2012; Warzecha et al., 2010).

The latest reports have also addressed how ESRPs not only down-regulated but also sometimes up-regulated in tumour progression. ESRPs proteins are low expressed in normal epithelial cells but increased in primary and advanced tumours. Remarkably, they decrease before the invasion area. The elastic change of ESRP level indicates their conflicting properties in tumour development. Constantly, it has been revealed that ESRPs inhibit mobility and proliferation of tumour cells while rescuing cells by improving

tolerance to reactive oxygen species (Hayakawa, Saitoh, & Miyazawa, 2017; Ishii et al., 2014).

1.4.3 *ESRPs splicing targets*

ESRPs switch splicing towards epithelial phenotype of about 200 genes, including FGFR2, NUMB, EXOC1, MAPK14, SCRIB and GSK3G (Dittmar et al., 2012; Warzecha & Carstens, 2012). In EMT, ESRPs are lost, their target genes splice differently which result in a different phenotype – the mesenchymal phenotype.

ESRP1 and 2 are master regulators of many AS events in epithelial cells where polarization is maintained by tight and adherent junctions. The cytoskeletons were destroyed by essential signalling pathways with actin cytoskeleton alteration in EMT. Meanwhile, the switches in cells promote focal adhesion and other structures that mediate cell motility forming. Some key proteins of these complexes and pathways were highlighted in Figure 1-13. mRNAs that change splicing during EMT or pathways that give epithelial and mesenchymal properties are shown in Figure 1-13. Therefore, this epithelial splicing regulatory network (SRN) switches the protein to epithelial isoforms to keep epithelial cell shape, activities and properties.

When EMT is activated, splicing is switched among hundreds of genes by inhibiting ESRP expression, resulting in proteomes that confer mesenchymal-like characteristics to cells. Three independent studies that aimed at fully identifying the splicing changes that occurred during EMT resulted in a great number of overlapping data sets (Shapiro et al., 2011; Warzecha et al., 2010; Y. Q. Yang et al., 2016). This is an important discovery and proposes that many gene pools share epithelial and mesenchymal cells, and yield different functions through AS. Therefore, this explanation has given many laboratories a motivation to consider and study the probability that these distinctly different functions of the same gene or protein might be the reflection of the result of the AS conversion (Warzecha & Carstens, 2012).

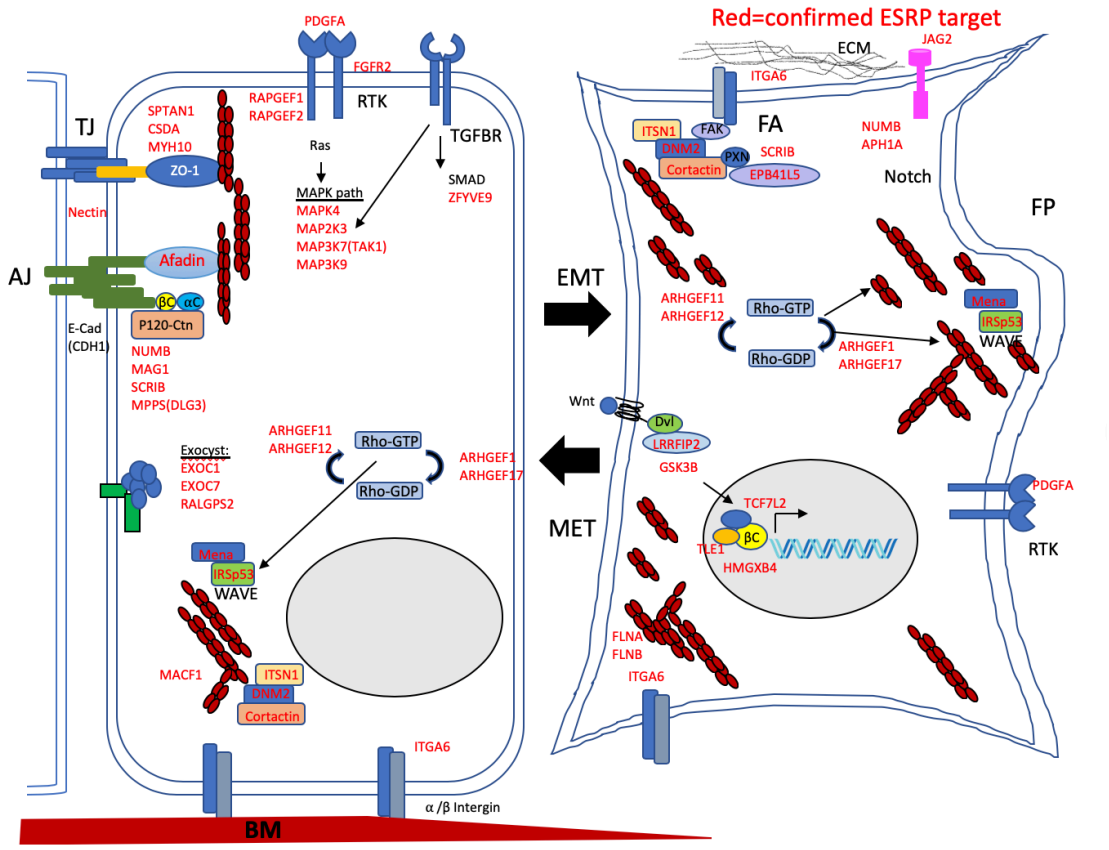


Figure 1-13 mRNAs that change splicing under control of ESRPs.

The left of the figure is an example of an epithelial cell while the right is a mesenchymal cell after EMT. Junction proteins that are important for polarization are shown. Other proteins important in signalling and interactions are shown. Other signalling and protein-protein interactions associated with EMT are also indicated. Adapted from (Warzecha & Carstens, 2012)

1.5 Fibroblast growth factor receptor 2 (*FGFR2*)

FGFR2 is well-known as a gene controlled by tissue-specific AS (Eswarakumar, Lax, & Schlessinger, 2005; Hovhannisyan et al., 2006; Y. Katoh & Katoh, 2009; Matsuda, Ueda, & Ishiwata, 2012; Muh, Hovhannisyan, & Carstens, 2002; Newman et al., 2006; Ranieri et al., 2016; Warzecha, Sato, et al., 2009). Recently, it has been acknowledged that ESRP1 and 2 were master modulators of FGFR2 AS, switching FGFR2 splicing to the epithelial phenotype.

FGFR2 gene abnormalities have been found in lung squamous cell carcinomas, breast cancers, endometrial cancers, and renal cancers, which are related to tumour development. About 10% of gastric cancer patients and 1% of breast cancer patients have *FGFR2* gene amplification. 3% of lung squamous cell carcinoma and 2% of endometrial cancer patients carry *FGFR2* gene mutations. It was indicated that increased *FGFR2* gene expression leads to a higher risk of breast cancer. Furthermore, the single nucleotide polymorphism of the gene is also correlated with breast cancer risk. FGFR inhibitors (for example, AZD4547, TKI258, etc.) show good anti-cancer effects on tumour cells carrying *FGFR2* gene amplification and gene mutations and can inhibit cell proliferation and promote apoptosis. *FGFR2* gene abnormality - amplification and/or mutation can be used as one of the reference factors for predicting the anticancer effect of FGFR inhibitors.

1.5.1 *The properties and function of FGFR2*

FGFR2 plays a significant role in embryogenesis and organ regeneration, particularly bone and vascular. The FGFR family functions through the bond of ligation and dimerization (pairing of receptors), triggering the tyrosine kinase domains to instigate a set of consequent signalling pathways (M. Katoh, 2016). Cell division, growth and differentiation are induced by these signalling pathways (Helsten, Schwaederle, & Kurzrock, 2015; M. Katoh, 2009; C. M. Lu et al., 2015; Tarkkonen et al., 2012). An increase in *FGFR2* IIIc inclusion and *FGFR2* IIIb exclusion has been indicated in the pathology related EMT (Thiery & Sleeman, 2006).

1.5.2 *FGFR2 isoforms and EMT*

The D3 region of *FGFR2* is encoded by three exons. Alternative splicing of the D3 region results in the C-terminus being highly variable, resulting in a high-affinity *FGFR2* IIIb or *FGFR2* IIIc subtype with a transmembrane structure (Eswarakumar et al., 2005). The two isoforms are mutually exclusive and highly tissue-specific (Figure 1-14) (Eswarakumar et al., 2005). Researchers observed a shift between *FGFR2* IIIb and *FGFR2* IIIc in EMT (Thiery & Sleeman, 2006). *FGFR2* IIIb is principally observed in epithelial cells while *FGFR2* IIIc is mainly located in mesenchymal cells (Orrurtreger et al., 1993). FGF7 and FGF10 expressed by interstitial cells can specifically activate *FGFR2* IIIb, while FGF2, FGF4, FGF6, FGF8 and FGF9 specifically activate *FGFR2* IIIc (Ornitz et al., 1996; X. Q. Zhang et al., 2006).

In epithelial cells, *FGFR2* IIIb functions in suppressing tumours, however *FGFR2* IIIc performs an oncogenic function. Based on research that the switching of *FGFR2* IIIb/ IIIc stimulates EMT, the consequence of the abnormal appearance of *FGFR2* IIIc in human epidermal cell that produces keratin indicated that, by morphological analysis, in reverse of *FGFR2* IIIb overexpression, the enforced expression and induction of *FGFR2* IIIc induce the epithelial cells to obtain a mesenchymal morphology and actin reformation. Furthermore, an increase of invasion and proliferation in *FGFR2* IIIc transfected keratinocytes was constant of its potential oncogenic function. All above biological changes illustrate that *FGFR2* splicing was complemented by regulation of EMT markers. Ultimately, the investigation of the distinguishing markers expression revealed that initiation of *FGFR2* IIIc prompts a procession related to the activation of the EMT associated with tumours, other than the EMT occurring during *FGFR2* IIIb-induced wound healing (Ranieri et al., 2016).

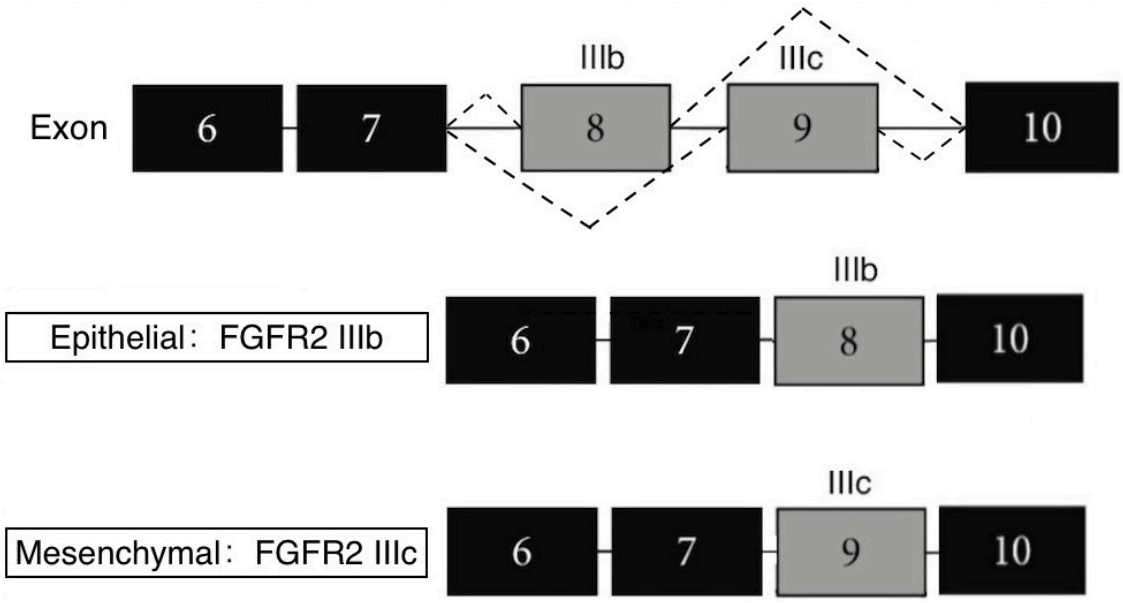


Figure 1-14 Splicing event of FGFR2.

Cells of epithelial phenotype skip exon 9/IIIc while in mesenchymal phenotype exon 9/IIIc is included.

1.5.3 *Regulation of FGFR2 alternative splicing*

FGFR2 has two mutually exclusive exons, exon8 /IIIb and exon 9/IIIc. The inclusion of exon IIIb in *FGFR2* IIIb or exon IIIc in *FGFR2* IIIc modifies the C-terminal half of the D3 loop of *FGFR2*, and is also responsible for the different ligand-binding specificities of the two isoforms (Eswarakumar et al., 2005). In recent years, a number of c*is*- and *trans*-acting elements have been identified that regulate the alternative splicing event. Exon IIIb suppression is mediated by the presence of weak splice sites flanking the exon, an ESS within the IIIb exon and both upstream and downstream ISS (Carstens, Wagner, & Garcia-Blanco, 2000; Delgatto & Breathnach, 1995; DelGatto, Gesnel, & Breathnach, 1996; Wagner et al., 2005; Wagner & Garcia-Blanco, 2001). Binding of hnRNPA1, PTB1, SR family proteins and other factors to these elements suppresses the exon IIIb and stimulates FGFR2 IIIc expression in mesenchymal cells (Carstens et al., 2000; Del Gatto-Konczak, Olive, Gesnel, & Breathnach, 1999; Wagner et al., 2005; Wagner & Garcia-

Blanco, 2001, 2002). In epithelial cells, employment of epithelial specific factors switches the splicing events to the inclusion of exon 8.

ESRP1 and ESRP2 are cell type-specific regulators of *FGFR2* splicing binding to an ISE/ISS-3 region within intron 8 to promote *FGFR2 IIIb* - specific splicing (Warzecha, Sato, et al., 2009). In the FGFR2 gene, there are some intronic and exonic *cis*-elements in and nearby exons IIIb and IIIc, in which some elements in intron 8 improve exon IIIb inclusion. ISE/ISS-3 is an ESRP binding site and could be disabled through dysfunction by mutants. ESRPs increase inclusion of exon IIIb and skip the exon IIIc by binding to ISE/ISS-3 (Figure 1-15 B). It is indicated that a partial switch from *FGFR2 IIIb* to *FGFR2 IIIc* that overlap with EMT properties, including reduction of epithelial markers like E-cadherin and Keratin 15, and rise of mesenchymal markers like TWIST, N-Cadherin and Vimentin correlated with the decrease of ESRPs expressing. This finding is shown by the investigation of *FGFR2* splicing in EMT stimulated by TWIST. (Warzecha, Sato, et al., 2009)

It has been investigated whether Fox-2 is also an inhibitor of exon IIIb and promotor of exon IIIc splicing. As Fox binding site and ISE/ISS-3 is nearby, the interaction between them might be required by both roles. However, there are numerous other proteins, such as hnRNP F, hnRNP H, and hnRNP M, that only inhibit exon IIIc without direct effects on exon IIIb inclusion (Hovhannisyan & Carstens, 2007; Mauger, Lin, & Garcia-Blanco, 2008). A complex of RBFOX2, hnRNPH1 and hnRNPF also contribute to epithelial-specific splicing by competing for binding to a site that is occupied by the SR proteins SRSF1 in mesenchymal cells (Baraniak, Chen, & Garcia-Blanco, 2006; Mauger et al., 2008). Other proteins and sequences have also been identified that contribute to the regulated expression of *FGFR2 IIIb* and *FGFR2 IIIc*, but the full details of the alternative splicing regulation remain to be worked out (Del Gato-Konczak et al., 2000; Hovhannisyan & Carstens, 2007; Muh et al., 2002; Newman et al., 2006).

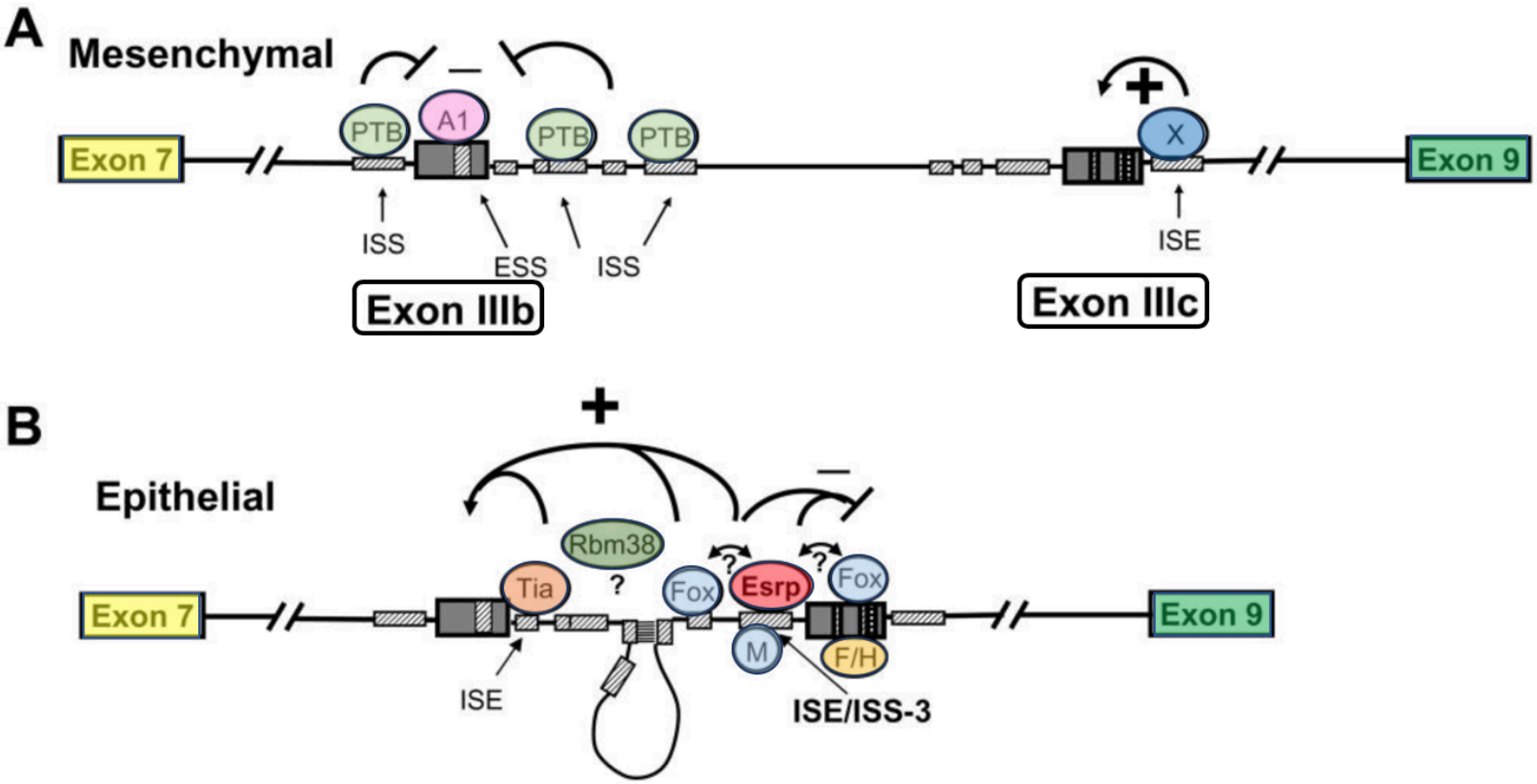


Figure 1-15 Schematic diagram for the regulation of FGFR2 Splicing in epithelial and mesenchymal cells

A Mesenchymal cell: Splice factors like PTB bind to cis-elements in the introns surrounding exon IIIb to inhibit the inclusion of exon IIIb. Some other unknown splice factors (X) bind to cis elements near exon IIIc to activate it.
B Epithelial cell: splice factors like Fox and Tia collaborate with ESRPs to inhibit the inclusion of exon IIIc and promote the inclusion of exon IIIb. Tia1 or TiaR; M, hnRNP M; F/H, hnRNP F or H. Adapted from (Warzecha, Sato, et al., 2009)

1.6 Using small molecules to modulate alternative splicing

Pre-mRNA splicing plays a crucial role in gene expression, while AS is an important procedure for the abundance of protein variations. AS is also vital to many diseases, especially cancer. One of the practical treatment options is to interfere with pre-mRNA splicing as some tumours are dependent on specific RNA splicing isoforms. Over the last few years, researchers have illustrated numerous RNA splicing regulators, and several of them have shown potential in disease treatment. However, the majority of splicing regulators target RNA splicing, which can lead to undesired side effects, so the options of single splicing selection have been detected (Newman et al., 2006; Warzecha, Sato, et al., 2009). Knowing the high incidence of abnormal splicing in tumours, small molecule regulators of RNA splicing signify capable new therapeutic strategies in the treatment of cancer (Salton & Misteli, 2016).

1.7 High-throughput screening and drug discovery

In order to find more effective drugs, pharmaceutical scientists and technologists have created a large number of new ways to discover new drugs. Especially with the constant progress of science and technology, the technical means are also continuously improved, new methods and new technologies are constantly reported (Michelini, Cevenini, Mezzanotte, Coppa, & Roda, 2010).

High-throughput drug screening uses screening models at the cellular and molecular levels. The results of these models are to be analysed on a case-by-case basis and need to be validated using additional tests. (Michelini et al., 2010)

Although the high-throughput screening model can reveal the pharmacological activity of the sieved substance at varying degrees, and even explain the mechanism of action and potency of the compound, it could not accurately explain the potential pharmacological or therapeutic effect due to the limitation of the target. For example, screening could show that a compound has a higher affinity for a particular receptor, but it cannot reveal the specific effect of the compound, neither can it distinguish whether it is an agonist or antagonist. In particular, for some targets that are not illustrated yet, although the interaction between the compound and the target can be

found, further research is needed for the evaluation of pharmacological action of the compound.

In summary, high-throughput screening as a means of discovery of new drugs with a wide range of options, lower screening costs, high sensitivity of assay (single molecule detection), high speed of assay, and reliable results, is an important means of new drug research. Through high-throughput drug screening methods, we can find innovative drugs with good effect, high clinical value and independent intellectual property rights.

1.7.1 *Bioassays for use in high-throughput screens*

Bioassays refer to the experimental method used to test the effect of drugs. Since high-throughput screening requires a small total reaction volume, and the response is highly specific and sensitive, higher sensitive screening models are also required. Commonly used screening models are established at the molecular level and cellular level to observe the interaction of drugs and molecular targets, which can directly understand the basic mechanism of action of drugs (Michelini et al., 2010). These models focus on receptors, enzymes, channels, and various cellular responses (Michelini et al., 2010; Seo et al., 2018). The drug screening model at the genetic level makes the drug screen range broader.

In recent years, there have also been drug screening models developed at the genetic level, making the drug screening model more extensive. In particular, various new screening models have emerged along with new achievements in basic research of life science such as telomerase inhibition, death induction, cell cycle inhibition, angiogenesis inhibition and other indicators of activity as a screening model of active ingredients of microorganisms. With the continuous elucidation of pathophysiological mechanisms, people pay more attention to screening mechanisms based on the mechanism of action. Especially with the progress of research on the human genome project, it is also very much concerned with the establishment of a screening model and a screening method for finding a certain gene as a target.

In addition, the use of cellular models or molecular-level models for the study and screening of compound toxicology and pharmacokinetics is also an important element in HTS. Screening of toxicology and pharmacokinetics through molecular cellular level

models is a very important step in the early assessment of drug in vivo processes and can reduce the upfront investment in the development of a large number of animal experimental drugs, in the evaluation of pharmacological effects and development of future significance.

Cell-based screening methods have many significant advantages over in vitro screening methods. Firstly, there is no need to purify the target protein, so there is no need to invest in a biologically active target. This advantage becomes more apparent as the number of drug targets available for treatment increases. It is difficult to establish a biochemical method for the identification of thousands of these new proteins in the absence of natural substrates. Secondly, the conformation, activity and biological function of the target protein in the cell are closer to their natural state. Thirdly, cell-based screening methods can quickly identify antagonistic compounds that are generally cytotoxic or compounds that do not reach the target within the cell across the cell membrane. Therefore, hits and lead compounds that have been screened have higher reliability. This method saves time and money in the drug development process.

1.7.2 *Examples of High-Throughput Screening derived therapeutics*

In recent years, researchers have identified more than a thousand potential protein biomarker molecules due to proteomics and genomics research and the tremendous progress made in the modelling of biological processes using computers. The list of biomarker candidates has also become longer and longer. However, most protein biomarker molecules proposed and documented in the literature are not used in clinical applications. The main reason that no progress has been made in the clinical development of new biomarker molecules has been the lack of a way to validate most biomarker candidates.

Based on a paper published in the journal *Science Translational Medicine* on July 11, 2012, lead author Ruth Hüttenhain and Martin Soste developed a strategy to measure potential biomarker molecules on a large scale and quickly validate them for clinical use (Huttenhain et al., 2012). This method is based on a high-throughput mass spectrometry-targeted technology that enables the determination of proteins present in a biological sample at a specific point in time in a reliable and reproducible manner.

In their study, the researchers used the developed method to detect 1,157 potential biomarkers: detecting their abundance changes in different human cancers and which ones are associated with mutations that promote cancer. Researchers eventually tested their assays in blood and urine samples from cancer patients and healthy individuals. They are capable of detecting more than 180 different biomarker molecules in serum, with a detection limit of one billionth of a gram of serum per ml. In urine samples, researchers found over 400 different protein biomarker molecules. With this approach, the list of potential biomarker molecules can be shortened quickly and effectively. Although these tests cannot be used directly in cancer diagnosis, they can shorten the gap between basic and clinical applications.

In the study of the identification of ovarian cancer, researchers confirmed their approach was valid: it was able to validate the potential biomarker molecules present in the serum. To do this, researchers can not only measure biomarker candidates described in the literature, but also measure new biomarker molecules predicted from computational models based on genomic data. Therefore, these findings underline the great potential of this mass spectrometric method to validate new protein biomarker molecules. The study also described the link between cancer, genetic data, and proteomic measurements that determine the patient's acute state. In summary, this new approach can be used to validate potential biomarker candidates in all patient samples and thus strategies for developing highly specific verification methods for disease-associated proteins can also be applied to other diseases.

Recently, researchers from Novartis Institute for Biomedical Research (H. Gao et al., 2015) established a large number of animal models of Patient-Derived Xenograft (PDX) for screening small-molecule anti-cancer compounds. This study shows that the PDX model is more effective in drugs of preclinical assessment and prediction of clinical outcomes has a very good application prospect.

In preclinical studies, there is still a lack of an ideal cancer model that can be used to characterize the effects of candidate therapies and methods in predicting the efficiency of clinical treatment. This shortage also exists in identifying the factors that cause heterogeneous responses to individualized treatment.

The PDX model is an in vivo model that transplants the patient's fresh tumour cells or tissues into immunodeficient mice and relies on the environmental growth provided by the mouse to preserve the primary tumour microenvironment and basic characteristics. Relying on this model can help with in-depth studies of tumourigenesis and the discovery of potential therapeutic targets, but also can help predict the patient's possible response to the drug, including efficacy, side effects, absorption and so on. In this study, Gao et al created about 1,000 PDX models that carry different cancer-driven genes and used these PDX models to perform small molecule compounds in vivo screening, evaluating the population response of 62 treatment strategies to 6 indicators happening.

By observing the association between phenotypes, drug response and drug resistance mechanisms, the researchers demonstrated that this method has good reproducibility and the ability to be transformed clinically. In addition, the study also showed that using the PDX model to assess the potential of clinical applications of some treatments is more accurate than cell line models. Taken together, this study shows that the PDX model has potential applications in preclinical assessment of cancer therapies and methods, and in predicting the outcome of clinical trials.

1.8 Hypothesis and aims

EMT and alternative splicing are the two processes focused on in this thesis. EMT is one of the hallmarks of cancer, so anti-EMT therapy is a potential treatment method for a variety of cancers. ESRPs is one of the most important splicing regulatory proteins associated with EMT and is an interesting protein with regards to driving alternative splicing to epithelial isoforms. Many studies have shown that ESRPs suppress cancer cell growth. Some of the pathways involved in regulating this splicing event have been elucidated.

This project aimed to find molecules able to reverse EMT and to explore whether they work through modifying ESRP activities. *FGFR2* is a well-known gene regulated by ESRPs and sensor of EMT. Splicing-sensitive fluorescent reporters based on *FGFR2* are sensitive tools to screen molecules for EMT activity. Splicing reporters provide a way to investigate an alternative splicing event by following the expression of fluorescent

proteins. A bichromatic *FGFR2* splicing reporter (pRGIIIc) was designed using the plasmid backbone of another splicing reporter and sequences from the *FGFR2* gene. (Figure 1-16)

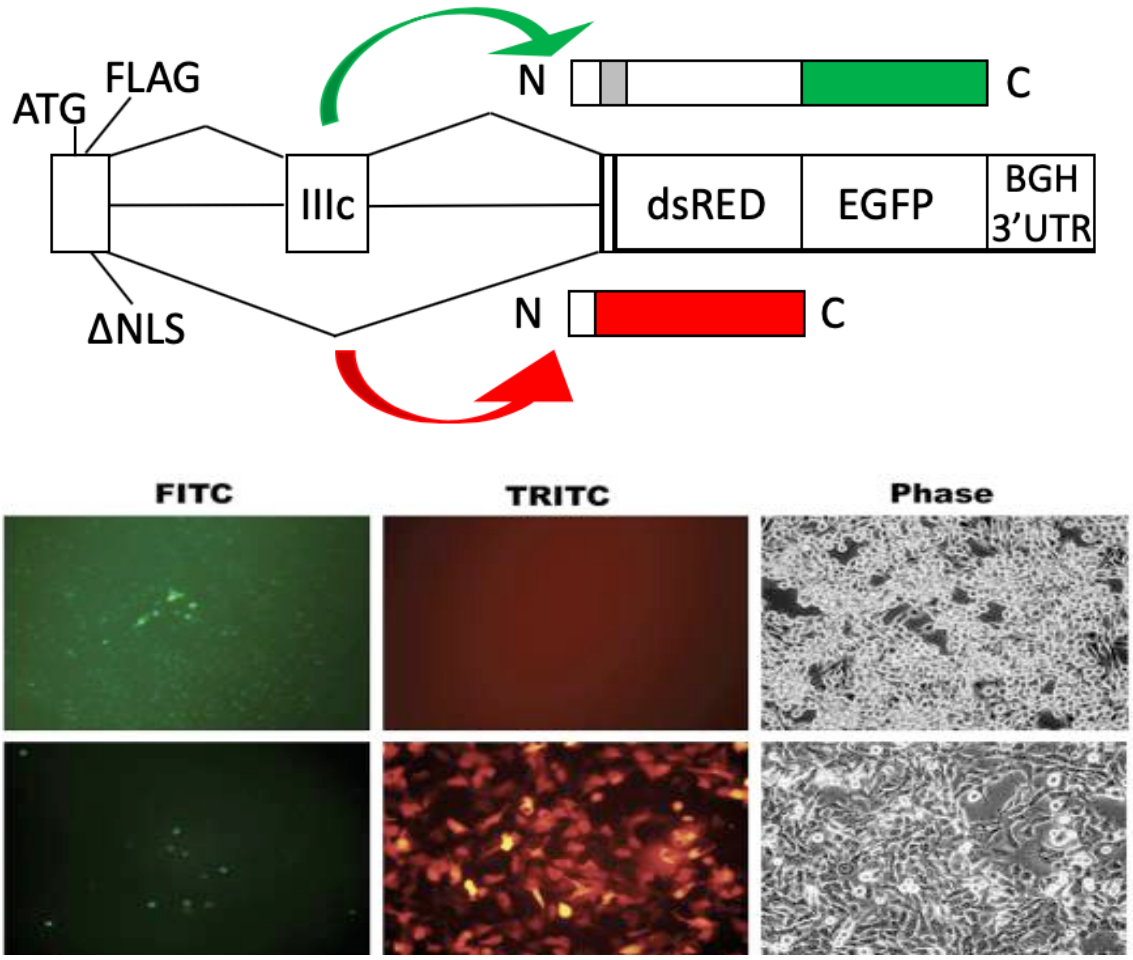


Figure 1-16 FGFR2 splicing-sensitive bichromatic fluorescent reporters on EMT and MET.

Upper: Design of the bichromatic reporter. A fusion EGFP protein is produced if exon IIIc of FGFR2 is selected and dsRED protein is expressed when exon IIIc is skipped.
Lower: AT3-RGIIIc and DT-RGIIIc cells imaged under immunofluorescence and phase contrast. Adapted from (Sebastian Oltean, Febbo, & Garcia-Blanco, 2008; S. Oltean et al., 2006)

The following aims were considered:

I. Explore whether ESRPs suppress tumour progression
II. Use of *FGFR2*-based splicing--sensitive fluorescent reporters as a molecular screening tool: screening for small molecules that can alter *FGFR2* exon splicing, promoting epithelial IIIb isoform production.
III. Test molecules which change *FGFR2* splicing for EMT activity *in vitro* and *in vivo*.
IV. Further investigate the activity of the small molecules to understand their mechanism of action in terms of EMT activity.

Chapter 2 Materials and methods

The methods and protocols described in this section have previously been described in my MSc thesis submitted to University of Bristol in 2013 entitled: Interplay of alternative splicing and epigenetic modifications in cancer cells.

2.1 Cell culture

2.1.1 *Cell lines*

HEK293 (Human embryonic kidney 293) is a cell line derived from human embryonic kidney cells grown in tissue culture, have been widely used in cell biology research for many years, because of their reliable growth and propensity for transfection. HEK293 cells are typical adherent mesenchymal phenotype cells that expressing completely *FGFR2* IIIc isoform. HEK293 cells were obtained from Microvascular Research Laboratories, University of Bristol, purchased from ATCC (ATCC® CRL-1573™).

PC3 is a human prostate cancer cell line which was established in 1979 from bone metastasis of grade IV of prostate cancer in a 62-year-old Caucasian male. PC3 cells have high metastatic potential compared to DU145 cells and LNCaP cells. PC3 cells are adherent cells which are more of mesenchymal phenotype and are often used in xenograft mouse model to study PCa. PC3 cells were obtained from Microvascular Research Laboratories, University of Bristol, purchased from ATCC (ATCC® CRL-1435™).

LNCaP clone FGC is a human prostate cancer cells that have low metastatic potential, that was isolated in 1977 derived from metastatic lymph node lesion of a 50-year-old Caucasian male with confirmed diagnosis of metastatic prostate carcinoma. LNCaP is a typical adherent epithelial cell line expressing high level of E-cadherin. LNCaP cells were purchased from ATCC (ATCC® CRL-1740™).

2.1.2 *Thawing cells*

Frozen cells were taken out from liquid nitrogen and shaken in a 37 °C water bath until thawed. The cell suspension was moved into a 15ml falcon tube and spun down at

1500rpm for 5 mins. The supernatant was aspirated, and the pellet was re-suspended with 5ml of pre-warmed medium and transferred to T25 flasks. Cells were cultured in a 37 °C incubator with 5% CO_2, the medium was refreshed every 3-4 days until the cells grew to 80-90% confluence.

2.1.3 *Sub-culture*

HEK293 cells were cultured in RPMI medium 1640 (1X) (Sigma, R8758) supplemented with 10% Fetal Bovine Serum (FBS, Sigma F2442) and 1% Penicillin streptomycin (5000 U/ml Penicillin, 5000µg/ml Streptomycin, Sigma, P4458).

PC3 human prostate cancer cells and LNCaP clone FGC human prostate cancer cells were cultured in RPMI medium 1640 (1X) supplemented with 10% FBS and 1% Penicillin streptomycin (see above concentration). All cell types detailed were incubated (unless specified otherwise) at 37 °C and 5% CO_2.

Cells were split when they reached 80-90% confluence. The medium was aspirated and 1x Phosphate Buffered Saline (PBS, diluted 1 in 10 from 10xPBS, Fisher, BP399-500) was added to the wash, then 0.25% Trypsin-EDTA (Gibco, 25200-056) (1ml /T75; 0.5ml/T25) was added. The flask was gently swirled and incubated at 37 °C for 3-5 minutes. Then the pre-warmed complete medium was added to stop the reaction. Cells at proper ratio were transferred to a new flask (e.g. 1:8).

2.1.4 *Freezing cells*

Following trypsinization protocol, freezing medium was prepared (2ml DMSO+8ml FBS) and mixed well. The suspension was aliquoted in two Falcon tubes and centrifuged at 1500rpm for 3 minutes (cell pellet should be observed after). The media was aspirated carefully without touching the pellet. 3ml of freezing media was added to each Falcon tube and mixed well. Suspensions aliquoted of 1.5ml/vial were stored in a freezing container at -80 °C and transferred to a liquid nitrogen tank after 1-2 days.

2.2 Transfection of cell lines

2.2.1 *Transient plasmid transfection*

ESRP1 and ESRP2 plasmids were used in transfection. ESRP1 plasmid (pIBX-C-FF-B-Esrp1-2A, obtained from Microvascular Research Laboratories, University of Bristol, see figure 2-1) was derived from the core vector pIBX-C-FF-B by sub-cloning cDNA of ESRP1 using the upstream *Stu*I site and downstream *Not*I site (Warzecha, Sato, et al., 2009). ESRP1 plasmid has a C-terminal flag and blasticidin resistant. ESRP2 plasmid (pBIGi-hESRP2, obtained from Dr Keith Brown, University of Bristol, see figure 2-1) was derived from the core vector pBIG2r by sub-cloning cDNA of ESRP2 and a flag tag using the upstream *BamH*I site and downstream *EcoR*V site. ESRP2 plasmid has a TET-on system and hygromycin resistant, with length of 725 amino acids and molecular weight of 78.36KDa.

PC3 cells were transfected using FuGene® HD Transfection Reagent (Promega E2311). PC3 cells were seeded in a 6-well plate at 200,000 cells per well. The following day, 2µg of the ESRP1 plasmid (or empty vector was diluted in 100µl Opti-MEM I Reduced Serum Medium (Gibco 31985-062). 6µl of the FuGene® HD Transfection Reagent was pipetted directly into the medium containing diluted plasmid to form the transfection complex, and then the complex was mixed and incubated at room temperature for 15 min. The transfection complex was added to cells and the wells swirled to ensure distribution. The cells were incubated for 48 hours before splitting to T25 flasks.

PC3 cells were transfected using FuGene® HD Transfection Reagent. PC3 cells were seeded in a 6-well plate at 200,000 cells per well. On the following day, 200ng of the ESRP2 TET-on inducible plasmid was diluted in 100µl Opti-MEM I Reduced Serum Medium. 6µl of the FuGene® HD Transfection Reagent was pipetted directly into the medium containing diluted plasmid to form the transfection complex, and then the complex was mixed and incubated at room temperature for 15 min. The transfection complex was added to cells and the wells swirled to ensure distribution. The cells were incubated for 48 hours before splitting to T25 flasks.

Stable PC3 ESRP1 overexpressing cells were transfected with inducible ESRP2 plasmids as above to construct PC3 ESRP1/2 overexpressing cell lines.

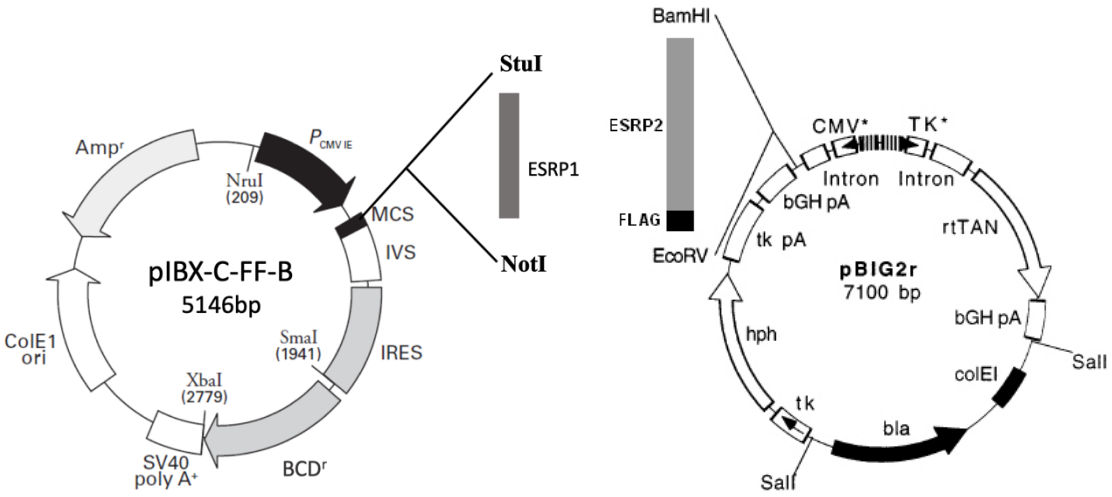


Figure 2-1 Plasmids maps

Left panel: *pBIX-C-FF-B-ESRP1-2A plasmid constructed by cloning cDNA of ESRP1 into pIBX-C-FF-B vector, between StuI and NotI.* ***Right panel:*** *pBIGi-hESRP2 plasmid constructed by cloning cDNA of ESRP2 and a flag tag sequence into pBIG2r vector, between BamHI and EcoRV.*

2.2.2 *Creating stably transfected cell lines*

48 hours after transfection with ESRP1 plasmid or ESRP2 inducible plasmid, the PC3 cells were split at several different ratios (e.g., 1:5, 1:10, 1:20) in complete medium containing the 10μg/ml Blasticidin (for ESRP1 plasmid) or 150μg/ml Hygromycin-B (for ESRP2 plasmid). For the next two weeks, replace the drug-containing medium twice a week. During the second week, some “islands” of the surviving cells were formed, and then large, healthy colonies was isolated using sterile pipette tips and split into new flasks and maintaining with selective medium for up to 5 weeks. The survived cells after selection were drug-resistant cells with transfected plasmids.

2.3 RNA assays

2.3.1 *RNA extraction*

Cells were lysed directly in the culture plate. 1ml of the TRIzol Reagent (Ambion, 18132201) was used per well for a six well plate. After the addition of the reagent, the cell lysate was passed 10 times through a pipette to form a homogenous lysate.

Samples were left standing at room temperature for 5 minutes to dissociate nucleoprotein complexes completely. After that, 0.2ml of chloroform was added per ml

of TRI REAGENT used, and samples were covered tightly and shaken strongly for 15 seconds. Samples were left to stand at room temperature for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4 °C. The mixture was separated into 3 phases: a red lower phase (containing protein), an interphase (containing DNA), and a colourless upper phase (containing RNA). The upper phase was moved to a clean Eppendorf tube, and then 0.5 ml of isopropanol was added per ml of TRIzol Reagent used in the first step and mixed.

The sample was left to stand for 10 minutes at room temperature, and then centrifuged at 12,000 rpm for 10 minutes at 4 °C. The RNA forms a pellet on the side and bottom of the tube. The supernatant was removed while the RNA pellet was washed by adding 1 ml (minimum) of 75% ethanol (v/v) per 1ml of TRIzol Reagent used.

Then the sample was vortexed and centrifuged at 10,000 rpm for 5 minutes at 4 °C. The RNA pellet was dried for 10 minutes by air-drying. The RNA pellet should not be dried completely as this would greatly reduce the solubility. 20µl of DEPC H_2O was added to resuspend the RNA pellet. Final extracted RNA was without DNA and proteins if the measured 260/280 ratio was ≥ 1.7.

2.3.2 *Treatment with DNase and cDNA synthesis*

2µg of RNA was used to make up to a final volume of 8µl by adding DEPC H_2O. 1µl of RQ1 Rnase-Free DNase 10x Reaction Buffer and 1µl of RQ1 Rnase-Free DNase (1 U/µl, final conc. 1U/1µg RNA) (Promega M6101) was added to each reaction and incubated at 37°C for 1 hour to degrade any genomic DNA contaminants. 1µl of RQ1 DNase Stop Solution was added and incubated at 65°C for 10minutes to terminate the reaction and inactivate the DNase.

The next step was to anneal DNA primers to the RNA template. 500ng of both oligo$(dT)_{15}$ (Promega, C1101) and random hexamer primers (Promega, C1181) were used. Primers were incubated with template at 95°C for 10 minutes and samples were then cooled on ice for 5 minutes.

After primer annealing, the second filament of cDNA was synthesised. 0.4mM dNTP mixture, 5µl M-MLV 5x reaction buffer and 200 units M-MLV reverse transcriptase

(M1701, Promega) were added to each reaction (in NO-RT control no M-MLV RT was added) and the total volume was made up to 25ul with DEPC water. cDNA production was completed via 90 minutes incubation at 37°C.

2.4 DNA assays

2.4.1 *DNA clean up*

DNA clean-up was done using QIAquick PCR Purification Kit (Qiagen 28104). The pH indicator I indicates a pH of ≤7.5 and was added to Buffer PB (1:250 volume). Buffer PB with pH indicator I was mixed with the PCR reaction at a 1:5 ratio. If the colour of the mixture is orange or violet, 10µl 3M sodium acetate (pH 5.0) was added and the mixture was mixed until the colour turned yellow. To bind DNA, the sample was applied to the QIAquick column which hung in a 2 ml collection tube and the tube with column was centrifuged for 30-60 seconds. The waste at the bottom of the collection tube was discarded and the QIAquick column was put back. After that, 750µl Buffer PE (100% ethanol was added up to the bottle volume label before use) was added to the QIAquick column and the column was centrifuged for 30-60 seconds to the wash. The waste at the bottom of the collection tube was discarded and the QIAquick column was put back. The QIAquick column was then centrifuged in the 2 ml collection tube for 1 minute to remove the remaining wash buffer. Each QIAquick column was put into a clean 1.5 ml Eppendorf tube. 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the centre of the QIAquick membrane to elute DNA and the column was left to stand for 1minute and then centrifuged for 1 minute.

2.4.2 *Polymerase Chain Reaction*

Polymerase chain reaction was used to amplify and demonstrate splicing pattern of *FGFR2* (Figure 2-2). Specific primers were designed to amplify both *FGFR2* IIIb and *FGFR2* IIIc transcripts. *β-actin* primers were used as a quantification control. The expected size of PCR products were 240 and 243 base pairs for IIIb and IIIc isoforms respectively, and 218 base pairs for *β-actin*. 25 µl of reaction mixture was made using 12.5 µl of 2xPCR MasterMix (Promega, M7502), 0.8 µlM of forward and reverse primer mixture and DEPC water to bring the reaction to a volume of 23µl. 2µl of cDNA was

added to each reaction and amplified using a thermal cycler. A water control with no template was needed. The primer sequences used were described in table 1. *β-actin* and *FGFR2* share the same PCR cycle condition described in table 2.

***FGFR2* Forward**	5'-AGAACGGCAGTAAATACGGG-3'
***FGFR2* Reverse**	5'-GGTAGTCTGGGGAAGCTGTA-3'
***β-actin* Forward**	5'-CATCCGCAAAGACCTGTACG-3'
***β-actin* Reverse**	5'-CCTGCTTGCTGATCCACATC-3'

Table 1: The primer sequences used for PCR

Step	Temperature (℃)	Time (min:sec)	Go to step	Cycle
1	95	5:00		
2	95	0:30		
3	55	0:30		
4	72	1:00	2	36
5	65	10:00		
6	4	Forever		

Table 2: PCR program cycle condition

2.4.3 *Digestion restriction enzyme treatment*

The two restriction digestion enzymes *Ava*I (NEB, R0152S) and *Hinc*II (NEB, R0103S) were used for digesting the two isoforms of *FGFR2* PCR products. *Ava*I only cuts *FGFR2* IIIb and *Hinc*II only cuts *FGFR2* IIIc. (Figure 2-2) 1µl of *Ava*I plus 1µl 1x CutSmart buffer (NEB, B7204S), and 1µl of *Hinc*II plus 1µl Ix NEBuffer™ 3.1 (NEB, B7203S) were added into 8µl *FGFR2* PCR products separately and incubated at 37 °C for 3 hours.

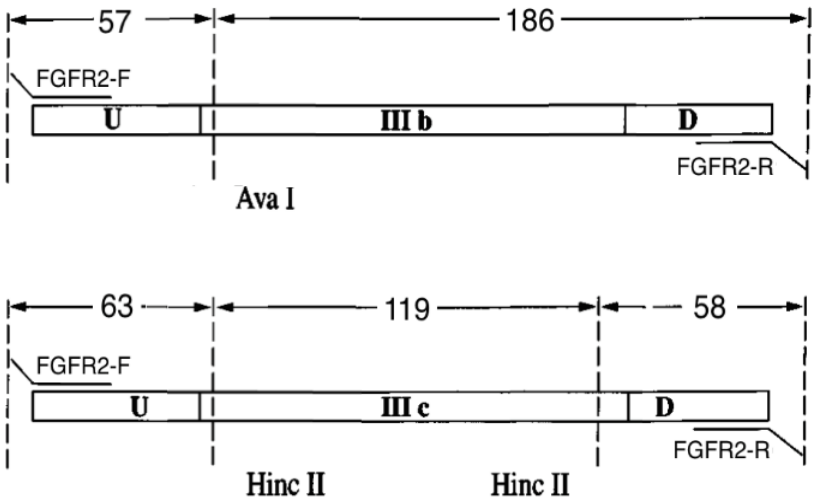


Figure 2-2 The maps of the RT-PCR products of FGFR2.

FGFR2 has two mutually exclusive isoforms FGFR2 IIIb and FGFR2 IIIc. As exon IIIb and IIIc have similar sizes, restriction digestion analysis is needed. I chose two digestion enzymes- AvaI and HincII: AvaI only cut FGFR2 IIIb and HincII only cut FGFR2 IIIc. PCR products have 243bp for FGFR2 IIIb which was cut into 57bp and for AvaI 186bp, and 240bp for FGFR2 IIIc cut into three pieces of 63bp, 119bp and 58bp. Adapted from (Carstens et al., 1997).

2.4.4 *Gel electrophoresis*

6 x loading buffer (Thermofisher, R1161) was added to each sample. PCR products were run on agarose gel at 90V for about 90 min. For making 2% agarose gel (w/v), 2g of agarose was mixed in 100ml 1xTAE (40mM Tris, 20mM Acetate and 1mM EDTA and typically has a pH around 8.6) plus 5µl SYBR Safe DNA gel stain (Thermofisher, S31102).

2.5 Protein assays

2.5.1 *Protein extraction*

- Protein extraction from cells

1x PBS and lysis buffer is prepared ice-cold and all work should be on ice. Cells were incubated on ice for 15 min to limit endocytosis (24-well plates in an ice bucket). Then the media was aspirated off. Cells were washed 2 times with 1ml/well of ice-cold 1xPBS. In the last wash all the PBS left was removed with a pipette. 80µl/well ice-cold lysis buffer was added (79.2µl of RIPA+ 0.8µl of protease inhibitor; 1X RIPA Buffer: 20 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1 mM Na2EDTA 1 mM EGTA 1% NP-40 1% sodium deoxycholate 2.5 mM sodium pyrophosphate 1 mM β-glycerophosphate 1 mM Na3VO4 1 µg/ml leupeptin) for each well. Cells were incubated on ice for 5 min (the plates were swirled occasionally for uniform spreading). Cells were scraped and gathered to one side by using a scraper while keeping the plates inclined to one side. The solution was mixed up and down several times by using a 200µl pipette for cell lysing. The lysate was transferred into a cold Eppendorf tube and incubated on ice for 20 minutes. Then the lysate was vortexed at maximum speed for 10sec every 5 minutes a total of four times.

Samples were centrifuged at 10000rpm for 10 min at 4 °C. Then the supernatant was collected and transferred to a fresh cold Eppendorf tube, aliquoted and stored at -20 or -80 °C.

- Protein extraction from tissues

Fresh tissue was frozen in liquid. Pre-cold lysis buffer (RIPA buffer with protease inhibitors) was added to the frozen tissues and mixed. The mixture was transferred in a homogenization tube and homogenized up and down slowly on ice until no solid was observed (add around 500µl every time and end up at around 10 µg/µl, between samples wash the tubes with water and then pre-cold PBS). The mixture was transferred into a cold Eppendorf tube. The homogenization tube was kept on ice all the time. The homogenized lysis was incubated on ice for 20 mins to maximize protein solubilisation and vortexed 10 seconds every 5 mins during the 20 mins. Then the sample was clarified

by centrifugation at 10000 RPM for 10 minutes at 4°C and the supernatant was moved to a fresh pre-iced tube.

2.5.2 *Total protein quantification*

0.1g of BSA (Bovine Serum Albumin, 4 °C) was weighted and dissolved in 10ml 1x PBS to get a stock solution in a concentration of 10mg/ml. The stock solution was diluted 1:10 (100µl in 900 µl of 1xPBS) to 1mg/ml.

Standards were made up for a 96 well plate:

-Eppendorf 1→ 700 µl (1mg/ml BSA)

-Eppendorf 2-7→350µl of 1x PBS+ 350µl of serial dilution

-Eppendorf 8→ 350µl of 1x PBS

Standard range was set as: 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0µg/ml of BSA.

10 µl of each standard was added per well into a 96-well plate in triplicate. The dilutions of the samples were made up at 1:50→ 1µl of sample+ 49µl of 1xPBS.

All the samples were kept on ice to prevent digestion from any proteinases. 10µl of each sample was added per well into a 96-well plate in triplicate. Bio-Rad Dye was diluted 1: 5 with 1xPBS (4ml of dye in 16ml of 1xPBS). 200µl of diluted dye was added to each well with a multichannel pipette. Then the prepared samples were left in incubation for 5 min. Analysis was run by Opsys MR Plate reader at 595nm (and 490nm) with 1 second shaking.

2.5.3 *Western blotting*

- Wet transfer

The samples were thawed on ice and protein concentration quantified; 30-50 µg was used per lane. Then 5x Protein Loading Buffer (Bioline, BIO-37045) was added in the samples and the mixtures were boiled for 5-10 min at 90 °C to break protein bonds. Pre-cast stain-free gels were loaded into the SDS-PAGE cassette with plates facing inwards; 1x SDS Running Buffer was added in the central reservoir, the combs were removed, and the lanes were rinsed with running buffer to remove debris. 5µl of Precision Plus

Protein™ Unstained Standards (Bio-Rad 1610363) and the samples were loaded. The gels were run at 150V until the blue dye of the samples was close to the bottom of the gel (1h). The running buffer level and whether the gels run properly need to be checked. After electrophoresis, the glass plate was lifted off and the stacking gel and the excess gel cut off. The gel was activated by Bio-Rad Image Lab and the proteins loaded were quantified to check whether they were equally loaded.

The gels were moved carefully to 1x Transfer buffer (Mix 100 ml of 10x transfer buffer, 200 ml of methanol, and 700 ml of ddH_2O, 10x transfer buffer recipe see table 3). Two pieces of sponge and 4 pieces of thick filter paper per gel were soaked in 1x Transfer buffer. The Immun-Blot™ polyvinylidene fluoride (PVDF) membrane (Bio-Rad, 1620177) was cut and activated by soaking in methanol for 5-10 seconds. Then the membrane was washed in distilled water and transferred to 1x Transfer buffer. The wet transfer was set up by placing the sponge, filter paper, PVDF membrane and gel into the clamp as below (Figure 2-3). Bubbles from each layer were removed gently by using the roller, and the clamps were placed into the reservoir and the buffer tank was filled with 1x Transfer buffer. (The black sides were put near the black side of the device.) The blot was run at 90V for 90 minutes, and then the gels were discarded. The total protein transferred to the membrane was relatively quantified by measuring lane and band relative quantities on the membrane with Image Lab™ Software (Bio-Rad).

The membrane was washed in TBS/Tween 0.3% (v/v) [3ml of Tween (Fisher, BP337-500) in 1000ml of 1x TBS], placed in a container on the rocking platform and covered with 5% BSA (w/v) in 0.3% TBS/Tween [2.5g of BSA in 50 ml of TBS/Tween] and left on the rocker for 1h at room temperature.

The primary antibody was made up of Mouse anti-E-Cadherin and Mouse anti-Vimentin both at 1:1000 in approx. 8ml of 5% BSA (w/v) in 0.3% TBS/T (v/v). The membrane was placed in a box with the primary antibody and left in incubation on a rocker in the DNA lab overnight at 4 °C. The membrane was moved into a new box the next morning and washed with TBS/Tween 0.3% (v/v) for 5 min five times.

The membrane was placed in a black box with the secondary antibody anti-mouse at 1:15000 for 1h at room temperature. The membrane was washed several times with TBS/Tween 0.3% (v/v) before running on the machine.

1ml detection reagent 1: Peroxide solution and 1ml detection 2: Luminol Enhancer solution (Pierce™ ECL western blotting substrate) was added to the membrane and mixed. After incubating for 5 mins the membrane was imaged by UVP ChemiDoc-It Imaging System.

10x Running Buffer:	**10x Transfer Buffer:**	**10x TBS Buffer:**
Tris Base (250mM): 30.4g	Tris Base (250mM): 30.4g	Tris Base (250mM): 24.2g
Glycine (1.92mM):144.2g	Glycine (1.92mM): 144.2g	NaCl: 80g
SDS: 10.0g		
Add ddH_2O to 1L	Add ddH_2O to 1L	Add ddH_2O to 1L and adjust PH=7.6 with HCl

Table 3: Recipes of Buffers used in Western Blot

Antibody	**Catalogue number**
Mouse anti-E-cadherin primary antibody	BD Biosciences, 612131
Mouse anti-Vimentin primary antibody	BD Biosciences, 550513
Rabbit anti-ESRP1 primary antibody	Sigma, HPA023719
Rabbit anti-ESRP2 primary antibody	Abcam, ab113486
Goat anti-Mouse IgG secondary antibody	Abcam, ab205719
Goat anti-Rabbit IgG secondary antibody	Abcam, ab205718
Alexa Fluro 488 Goat anti-Mouse IgG (H+L)	Invitrogen, A11001

Table 4: Antibodies used in Western Blot and Immunofluorescence

- Semi-dry transfer for ESRP1 detection

Gel electrophoresis was done as described above, except that the gel was run at 100V until the blue dye of the samples was close to the bottom of the gel (90mins). The running buffer level and whether the gels ran properly were checked. After electrophoresis, the plate was lifted off and the stacking gel and the excess gel were cut off. The gel was activated by Bio-Rad Image Lab and the proteins loaded were quantified to check whether they were equally loaded.

The gels were moved carefully to 1x Transfer buffer. Two pieces of sponge and two pieces of thick filter paper per gel were soaked in 1x Transfer buffer. The Immuno-Blot™ PVDF membrane was cut and activated by soaking in methanol for 5-10 seconds, then washed in distilled water and transferred to 1x Transfer buffer. The semi-dry transfer blotter was set up by placing the sponges, filter papers, PVDF membrane and gel into the clamp to construct a gel "sandwich" (Figure 2-4). The membrane was put on the anode (bottom) side relative to the gel. The semi-dry transfer kit was closed, and the power supply connected to transfer at constant AMPs (0.8mA/cm^2), not volts, for 75 min.

The membrane was washed in TBS/Tween 0.1% (v/v) [1ml of Tween in 1000ml of 1XTBS], placed in a container on the rocking platform and covered with 5% BSA (w/v) in 0.1% TBS/Tween (v/v) [2.5g of BSA in 50 ml of TBS/Tween] and left on the Rocker for 1~2 hours at room temperature.

The primary antibody was made up of rabbit anti-ESRP1 (see table 4) at 1:1000 in 10ml of 5% BSA (w/v) in 0.3% TBS/T (v/v). The membrane was placed in a box with the primary antibody and left for incubation on a rocker in the cold room over 24 hours at 4 °C. The membrane was moved to a new box the next morning and washed with TBS/Tween 0.3% (v/v) five times and for 5 minutes each time.

The membrane was incubated with the secondary antibody anti-rabbit IgG (see table 4) at 1:10000 for 1~2 hours at room temperature. The membrane was washed several times with TBS/Tween 0.3% (v/v) before running on the machine.

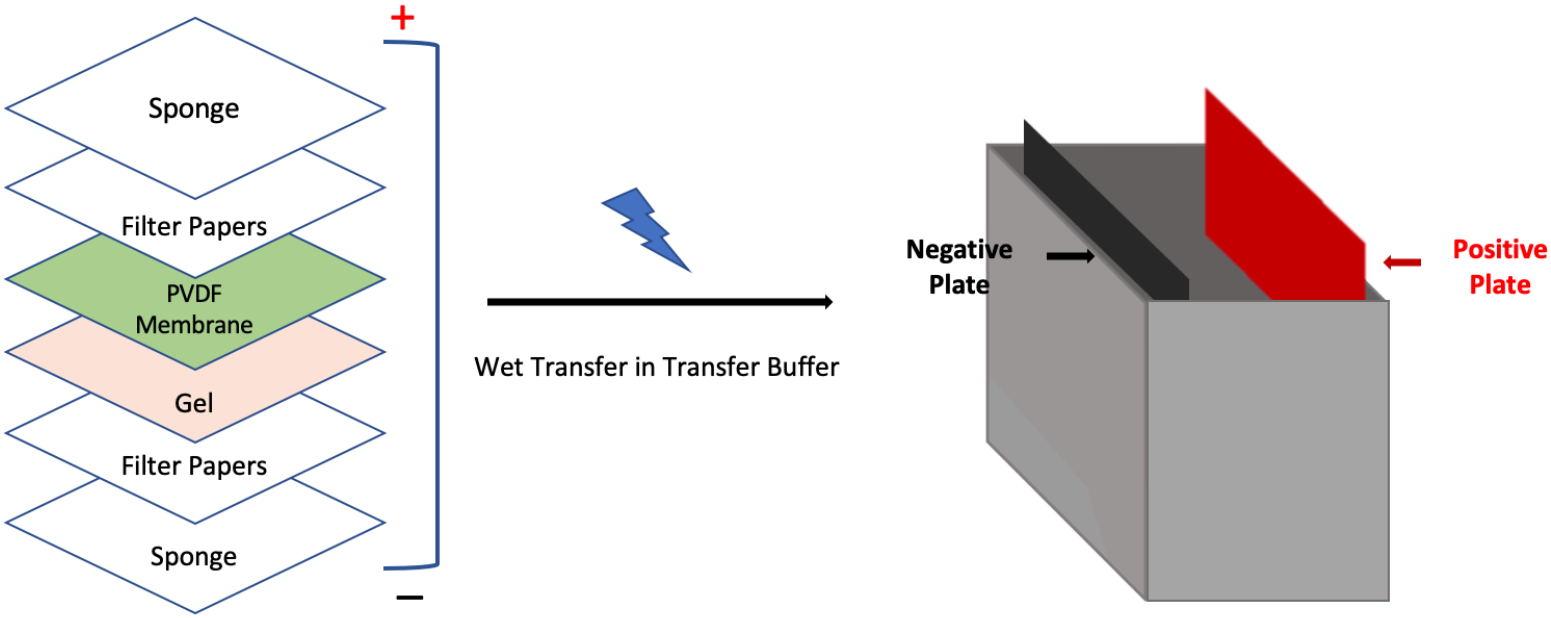


Figure 2-3 Diagram of a classical wet transfer blotter.

Between two plates (positive and negative), the following are placed: the sponge, filter papers, PVDF membrane and gel, and another layer of filter paper. The sandwich was inserted into the transfer tank with transfer buffer and the positive plate is on the anode side while the negative plate is on the cathode side. Adapted from (Mahmood & Yang, 2012)

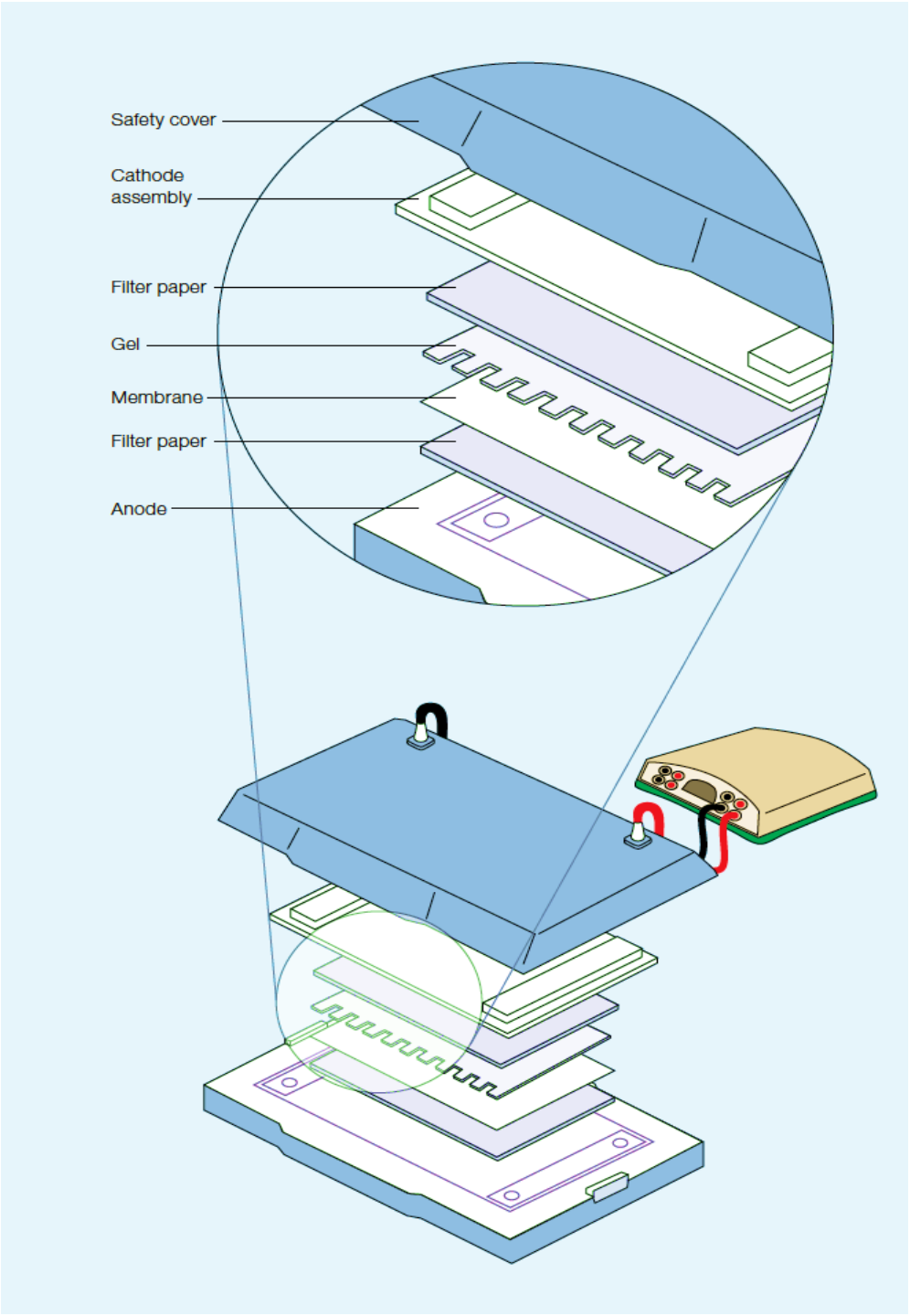


Figure 2-4 General set-up of a semi-dry transfer.

Between two plates (anode and cathode) connected to a power source, the following are placed: filter paper, membrane, gel and filter paper. (Adapted from Bio-Rad website)

2.6 Bacterial transformation

Agar and LB broth (Agar: Agar packet + 500ml dH_2O; LB Broth: 10g LB broth + 500ml dH_2O) were prepared and autoclaved at 121 °C for 15 minutes. Once the agar had cooled down, 100 µg/ml ampicillin was added and mixed. The mixture was poured into plates (10-15ml/plate) with lids on and left to set. Bunsen burner flame was kept on while pouring and setting plates.

Plates kept at 4 °C were warmed in an incubator while DH5α bacteria were thawed on ice. 1µl plasmid was added to 30µl bacteria then mixed by stirring. The mixture was put on ice for 30 minutes and heat-shocked at 42 °C for 30 seconds. After that the mixture was iced for 2 minutes. 300µl of SOC media was added to bacteria and the mixture was shaken for 45 minutes at 37 °C.

Warm plates were moved out of the incubator. Pasteur pipette was sterilized using ethanol and the Bunsen burner. 50µl of bacteria was pipetted onto a plate and spread with the Pasteur pipette. Plates were incubated at 37 °C overnight.

5ml of LB broth was put in a Falcon tube with ampicillin 100µg/ml. A single colony on the plate was touched using a sterile tip and then dropped in LB broth. The lids were loosely taped on the Falcon tubes and put in a shaker overnight at 37 °C.

Plasmid was extracted using QIAprep Spin Miniprep Kit (Qiagen, 27106). Broth from the Falcon tube was aliquoted into 1.5ml Eppendorf tubes and then centrifuged for 1 min at 13000 rpm. The supernatant was discarded. Pellet was re-suspended in 250µl P1 buffer (P1 kept at 4 °C) and mixed thoroughly by pipetting up and down. 250µl P2 lysis buffer was added into the resuspension and tubes were gently inverted 6 times. Then 350µl N3 buffer was added and tubes were gently inverted 6-8 times. Then the mixture was spun for 10 minutes at 13000 rpm. Supernatant was transferred to the filter tube and spun for 1 min to allow plasmid binding to filter, and then the waste at the bottom of the collection tube was aspirated. Then the filter tube was washed with 750µl PE wash buffer, spun for 1 minute and the waste at the bottom of the collection tube was aspirated. Filter was put into a clean Eppendorf tube and 50 µl of EB elution buffer was added; the mixture was left to stand for 1 min and spun for 1 min. The liquid at the bottom of the tube was pure plasmid. Plasmid concentration was measured using the

NanoDrop™ 2000/2000c Spectrophotometers (Thermofisher, ND-2000, a full-spectrum, UV-Vis spectrophotometers used to quantify and assess purity of DNA, RNA, Protein and more).

2.7 Chemical screen

2.7.1 *LOPAC primary screen*

HEK293 pRGIIIc (FGFR2 exon IIIc cloning in RG6 minigene) cells were used as reporter cells. 10,000 cells/well of HEK cells were seeded in a 96-well plate and incubated at 37 °C with 5% CO_2. After 24 hours, cells were treated with compounds from LOPAC Library at 10 µM. After 48 hours of treatment, the plate was read by a VICTOR plate reader. Data was analysed by One-way ANOVA using GraphPad Prism 7 (GraphPad, Prism 7 for Mac OS X, a commercial scientific 2D graphing and statistics software).

2.7.2 *Elimination of false positives in the control screen*

PC3 DSS (pRG8ab distal splice site control reporter) cells were used as GFP control and PC3 PSS (pRG8ab proximal splice site control reporter) cells were used as RFP control. The control plasmid DNA did not contain the intron of the original pRG8ab (VEGF exon 8ab cloning in RG5 minigene) reporter, therefore, mimicked the mRNA transcripts that are produced when either a proximal or distal reporter 3' splice site is chosen. (Figure 2-4) The plasmids were termed PSS and DSS control reporters. Both control cell lines were treated as above, and the data was analysed as above as well. Then the analysed data was combined with the data from the primary screen to eliminate the false positives. Data was analysed by One-way ANOVA using GraphPad Prism 7.

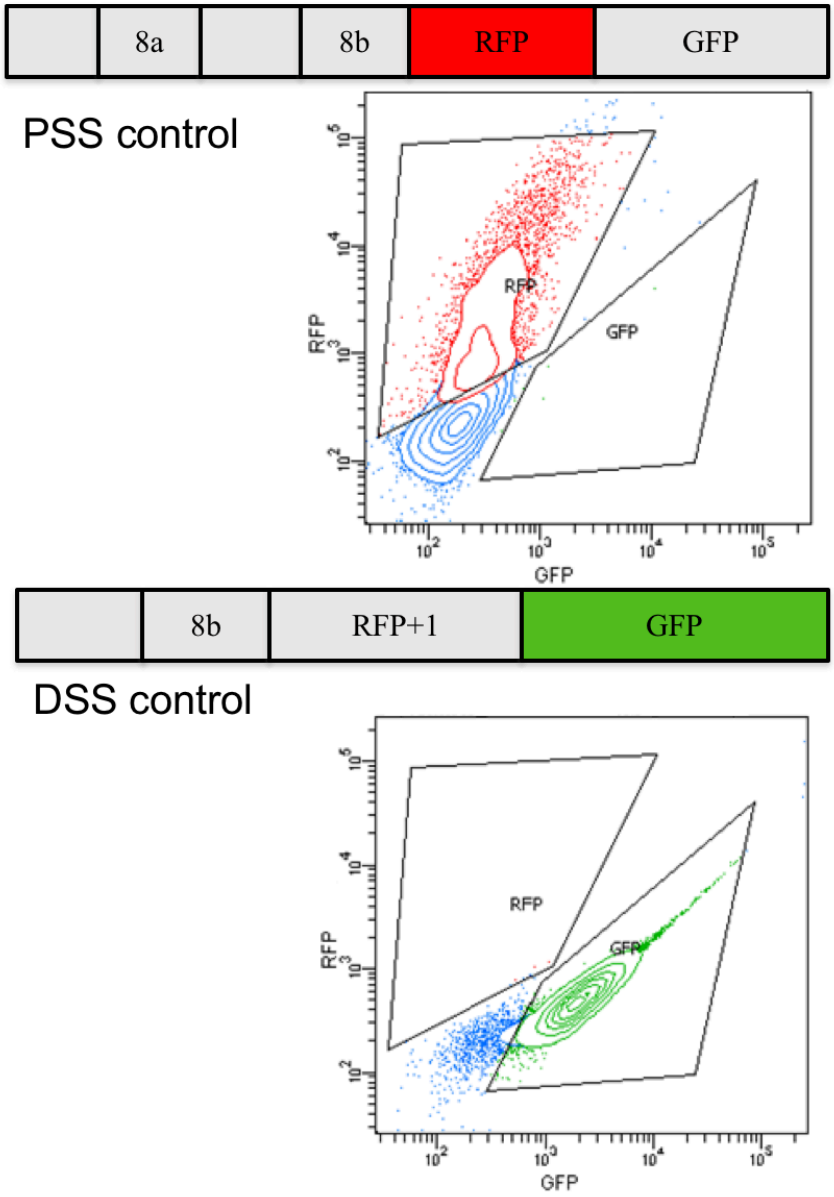


Figure 2-4 Design and expression of proximal splice site and distal splice site control reporters.

DSS and PSS control reporters were constructed to mimic the two mature mRNA transcripts that can be produced from the splicing of pRG8ab pre-mRNA - proximal (PSS) and distal splice site (DSS) controls. Both reporters were transfected into PC3 cells individually and their expression was confirmed by FACS. (by Dr. Eleanor Star, previous PhD student in our group).

2.8 *In vitro* cell assays

2.8.1 *Cell growth curve*

PC3 cells were seeded with 100,000 cells per well in eight 12-well plates, and then treated with chemicals 24 hours after seeding. The medium with chemicals was refreshed every 48 hours after first treatment. Cells were counted every 24 hours after seeding in the plate. All data was calculated and analysed with Prism and tested by two-way ANOVA. All the treatments had 12 repeats.

PC3 with control, ESRP2, and ESRP1/2 overexpressing cell lines (plasmids used see figure 2-1) were plated 100,000 cells per well (depending on cell type) in eight 12-well plates, and then treated with Doxycycline to induce ESRP2 expressing 24 hours after seeding. The medium with Doxycycline was refreshed every 48 hours after first treatment. Cells were counted every 24 hours after seeding in the plate. All data was calculated and analysed with Prism and tested by two-way ANOVA. All the treatments had 4 repeats.

LNCaP cell lines were plated 100,000 cells per well in eight 12-well plates. The medium was refreshed every 48 hours and cells were counted every 24 hours after seeding in the plate. All data was calculated and analysed with Prism and tested by two-way ANOVA. All the treatments had 4 repeats.

2.8.2 *Cell migration assay – wound healing assay*

Cells were pre-treated with chemicals in T75 flasks for 48 hours and 500,000 cells plated per well in 6-well dishes. After an overnight starving, the cell monolayers were washed with pre-warmed PBS (5 minutes, twice), scraped across using a P-20 micropipette tip and a fresh medium with chemicals added to the wells. The initial gap area (0 h) and the residual gap area at 24h and 48h after wounding were calculated from photomicrographs and then analysed by Image J and Prism using two-way ANOVA. All the treatments were done in triplicates.

2.8.3 *Cell migration assay – Boyden chamber assay*

Cells were pre-treated with chemicals for 48 hours and starved overnight. After that cells were trypsinized, collected with serum free medium in 15ml Falcon tubes and spun down. Then the cells were re-suspended with serum-free medium, counted and seeded with 150,000 cells per 300 µl in inserts in a 24-well plate (Figure 2-4). A normal complete medium with 10% FBS Medium was placed in the well outside the inserts. Then the plates were incubated at 37C for 24 hours. The cells that migrated through the membrane at 24h after seeding were stained with DAPI and then counted from photomicrographs. The count number was analysed by Prism using two-way ANOVA. All the treatments had four repeats.

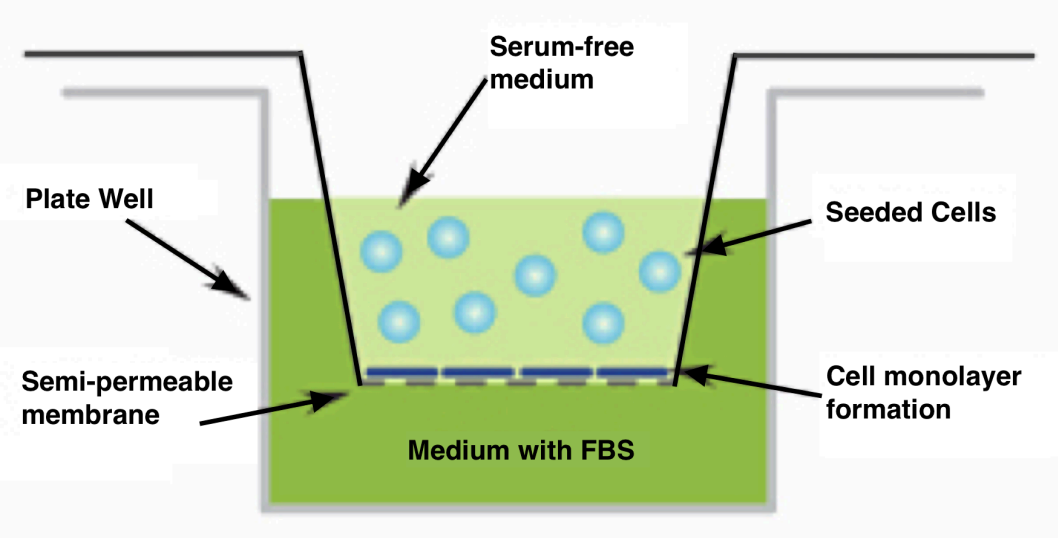


Figure 2-5 Schematic diagram of experimental setup of Boyden Chamber assay.

An insert is placed in the well; the insert has a semi-permeable membrane. Cells in a serum-free medium or low-serum medium are seeded in the insert and then travel through the membrane to the other side. (Adapted from Merck Millipore website)

2.8.4 *Proliferation assay – MTT assay*

MTT assay was done using Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen V13154). Before the assay, 12 mM MTT stock solution was prepared by adding 1 mL of PBS to the 5 mg tube of MTT (Component A), and 10 mL of 0.01 M HCl was added to the tube containing 1 gm of SDS (Component B, which should be used promptly once prepared). The solutions A and B were gently mixed by inversion or sonication until

dissolved, respectively. All the experiment groups were pre-treated with either control (DMSO) or one of different doses of the three drugs (LLSOs) for 48 hours (for cell to reach the optimized measuring density $1*10^6$) before seeding. After pre-treatment, PC3 cells were seeded in a 96-well plate at 10000 cells per well. The next day, the medium was removed and 100 µl of fresh complete medium per well was added. 10 µl of 12 mM MTT stock solution per well was added and mixed well. Meanwhile, the same solution was added to wells with 100 µL of medium without cells as a negative control. Following a 4 hours incubation at 37 °C, 100 µL of the SDS-HCl solution per well was added and mixed carefully with pipettes. Finally, after another 4 hours incubation at 37°C, the absorbance at 570nm for each well was measured by Absorbance Microplate Readers from BMG LABTECH. Three repeats were applied for each cell line.

2.8.5 *Immunofluorescence (E-cadherin)*

Cells were split onto coverslips in a 12-well plate at 300,000 cells per well with 2ml pre-warmed medium per well. Chemicals were added in wells 24 hours after seeding. During the experiment, cells were incubated in 37°C incubators with 5% CO_2. After another 48 hours, cells were washed with PBS and fixed with 1.5ml 4% PFA per well for 10 minutes. After 5 mins washing twice with 1xPBS, cells were permeabilized with 1xPBS-TritonX (0.05%) (v/v) for 10 mins. Then cells on the coverslips were washed again with 1xPBS for 5 minutes twice and blocked with 1% BSA (w/v) + 5% normal goat serum in PBS (v/v) for 1 hour. After blocking, cells were incubated with a primary antibody in 1% BSA in PBS at 1:500 dilution overnight at 4 °C. After washing twice for 5 minutes with 1xPBS, cells were incubated with Alexa Fluor secondary antibody diluted in 1% BSA in PBS at 1:1000 at room temperature for 1 hour. After two 5 mins washings with 1xPBS, cells on the coverslips were put onto slides with a drop of Vectashield mounting medium containing DAPI (VECTASHIELD® Antifade Mounting Medium with DAPI, Vector Labs, H-1200) (cell-side down). The coverslips were sealed with nail polish and then imaged under fluorescence microscopy.

2.9 *In vivo* models

2.9.1 *Subcutaneous xenograft models*

PC3 with control, ESRP1, ESRP2 and ESRP1/2 overexpressing cells (see section 2.2) were cultured in media containing 2.5μg/ml Doxycycline or DMSO for 48 hours to induce ESRP2 expression. Cells were detached from culture flasks and diluted in pre-cold PBS to a density of $1x10^7$/ml and placed on ice. 100 μl of cell suspension was subcutaneously injected into each right flank of CD1- nude mice (Charles River). Tumour sizes were measured by calliper two times a week. Mice were culled by cervical dislocation (Schedule 1) when the first tumour size reached the maximum allowed by our project licence (12mm x 12mm) and the tumours were extracted. Images of each tumour were taken and weighted. Tumours were flash frozen in liquid nitrogen. 1ml of Trizol was added to each tumour sample, which were homogenised for RNA extraction as described.

For treatment studies, PC3 human prostate cancer cells were detached from culture flasks and diluted in pre-cold PBS to a density of $1x10^7$/ml and placed on ice. 100 μl of cell suspension was subcutaneously injected into each right flank of CD1- nude mice, 18 mice in total. Tumour sizes were measured by calliper two times per week. When the tumour size reached 3mm x 3mm, 10μM LLSO02 (one of the three hit compounds selected from LOPAC screen. Nemadipine-A, a L-type calcium channel protein inhibitor), 50μM LLSO02 or DMSO were injected intraperitoneally two times a week, 6 mice per treatment group. Mice were culled by cervical dislocation (Schedule 1) when the tumour size reached a maximum (12mm x 12mm) and the tumours were extracted. Each tumour was imaged and weighed. Tumours were flash frozen in liquid nitrogen for further analysis.

Tumour volumes were estimated using the formula 'volume = [(length + width)/2] x length x width'. Quantitation of the tumour volumes was analysed by Two-way ANOVA using GraphPad Prism7.

2.10 Statistical analysis

Comparisons of two datasets at a single timepoint were carried out using Students' T-test or Mann Whitney test. Comparisons of three or more datasets at a single timepoint were by one-way ANOVA with Dunnett's post-test. A comparison of three or more groups at multi-timepoints was performed using two-way ANOVA. $P<0.05$ was considered a statistically significant difference.

Chapter 3 ESRPs functions in tumour progression

Data presented in this chapter was published as part of publication "Androgen-regulated transcription of ESRP2 drives alternative splicing patterns in prostate cancer." eLife 2019;8:e47678 DOI: 10.7554/eLife.47678

3.1 Introduction

Epithelial splicing regulatory proteins (ESRP1 and ESRP2) switch splicing to epithelial phonotype during EMT (Warzecha, Shen, et al., 2009). EMT is a procedure that involves polar epithelial cells transforming into stromal cells and is essential in normal development of the body and the invasion and metastasis of tumour cells. ESRPs switch numerous genes splicing towards the epithelial phenotype. In EMT, ESRP is lost; those genes splice differently which results in a different phenotype - mesenchymal phenotype. Recently, lots of experimental data indicate that epithelial splicing regulatory proteins (ESRPs) have the effect of inhibiting tumour EMT, are negative regulators of malignant transformation of tumours and are regulated by TGF-β/Smad signalling pathway. ESRPs directly or indirectly regulate the proteins related to EMT such as FGFR2, Rac1b, E-cadherin and ZEBs at the post-transcriptional level by AS to inhibit EMT. Therefore, it is important to find what regulates ESRPs activity.

3.2 Results

3.2.1 *The effects of ESRPs in prostate cancer cell growth and EMT markers in vitro*

Given that ESRPs have the effect of inhibiting tumour EMT, we hope to reveal the possible mechanism of ESRPs inhibiting EMT in tumour cells.

First of all, we tried to figure out how ESRPs affect the prostate cancer cell line –PC3 cells functions. I constructed PC3 ESRP1 overexpressing cells by transfecting ESRP1 plasmids into PC3 cells and then selected stable clones by culturing cells in selective medium with 10μg/ml Blasticidin, PC3 ESRP2 overexpressing cells by transfecting ESRP2 plasmids into PC3 cells and then selected stable clones by culturing cells in in selective medium with 150μg/ml Hygromycin, and PC3 ESRP1/2 overexpressing cells by

transfecting ESRP2 plasmids into stable PC3 ESRP1 overexpressing cells and then selected stable clones by culturing cells in in selective medium with 150μg/ml Hygromycin(described in Materials and Methods, 2.2). As ESRP2 plasmid is a TET-on inducible vector, ESRP2 was induced by 2-5μg/ml doxycycline for 48 hours. After ESRPs overexpression was confirmed by Western blot (Figure 3-1), a series of cell functional assays were carried out.

The effect of ESRPs on cell growth was investigated by the cell growth curve (Figure 3-2). Cells were seeded at 100,000 per well density in a 12-well plate, four repeat wells were used for control and each engineered cell line. Cells were counted every 24 hours and the media were changed every 48 hours. As a result, both cell lines – PC3 overexpressing ESRP1 only and ESRP2 only, showed a significant cell growth decrease compared to the control cell line. Moreover, PC3 overexpressing both ESRP1 and ESRP2 showed a greater reduction on cell growth, which may be the combined effect of ESRP1 and ESRP2 overexpression.

Furthermore, the effect of ESRPs on EMT markers was validated by immunofluorescence analysis. PC3 cells with ESRP1/ESPR2/ESRP1&2 overexpression were cultured on coverslips and cells were incubated for a further 48 hours. Triplicate wells were used for control and each engineered PC3 cell line. The coverslips were fixed and stained with a fluorescent antibody against E-cadherin, an epithelial cell marker. PC3 cells stained with mouse IgG instead of primary were used as negative control and LNCaP cells were used as a positive control. PC3 ESRP2 and PC3 ESRP1/2 cells were grown in a medium supplemented with 2.5μg/ml doxycycline for 48 hours prior to staining to induce ESRP2 expression. The immunofluorescences analysis of the EMT-marker – E-cadherin confirmed that ESRPs not only increased E-cadherin expression level, but also changed the localization of it in PC3 cells (Figure 3-3). This result, in part, validates the previous publication's statement that ESRPs inhibit tumour EMT.

Finally, whether or not ESRPs will decrease cell migration in PC3 cells was revealed by Boyden chamber assay (Figure 3-4). Boyden chamber assay was performed 48 hours after ESRP1, ESRP2, ESRP1&2 overexpressing PC3 cell lines and empty vector control PC3 cell line were seeded in the inserts. Four repeats were applied for each cell line. All the four experimental cells were grown overnight in a reduced medium with 2% FBS for

starving cells before seeding in the inserts. PC3 cells not starved were used as negative control. None of the overexpressing cell lines showed any change for PC3 cell migration rate.

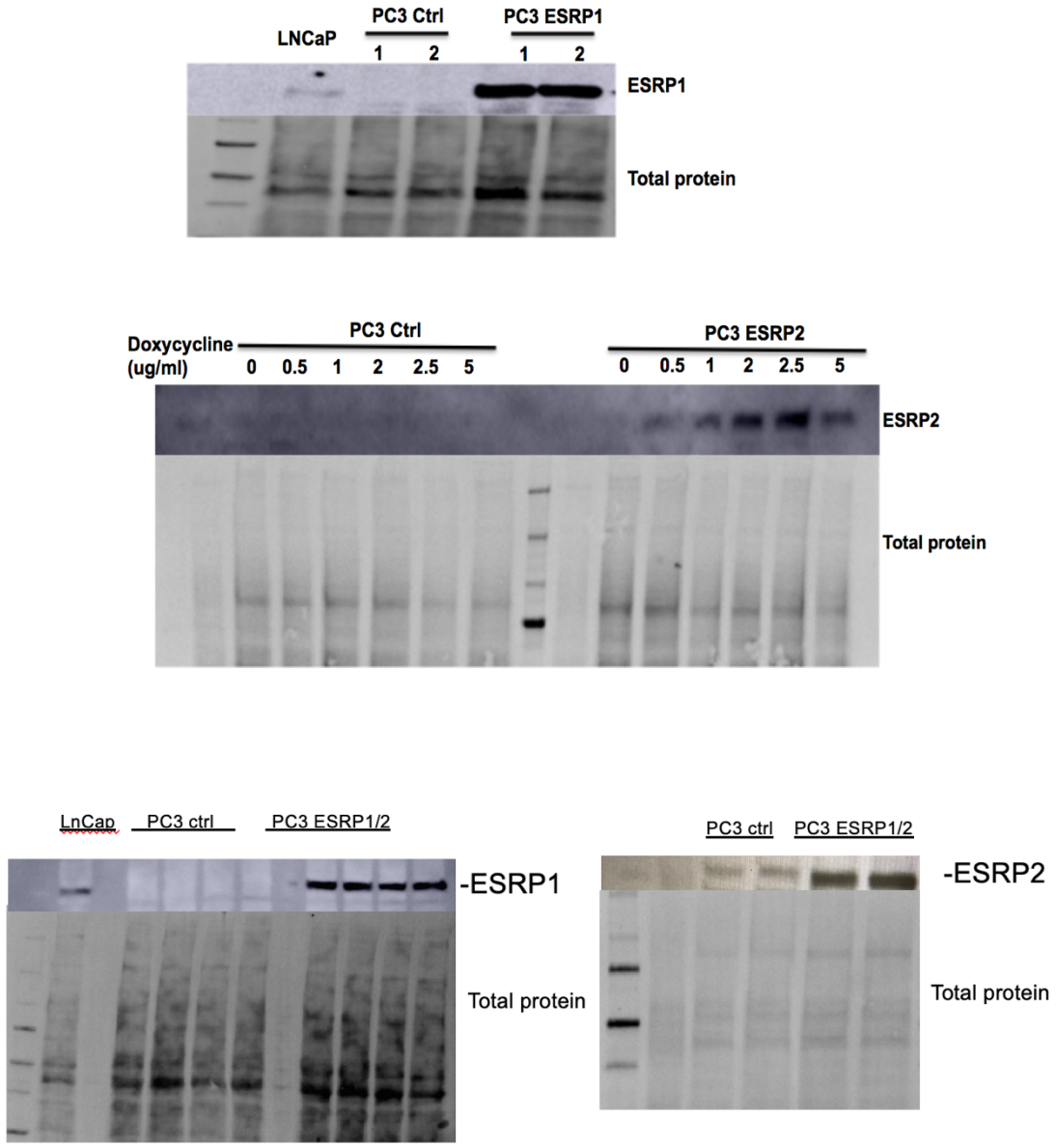


Figure 3-1 Validation of ESRPs transfection in PC3 cells and optimization of ESRP2 induction.

Western blot analysis was carried out after transfection of ESRP1, ESRP2 and empty vector plasmid in PC3 cells. Total protein blot was used as an internal control. ***Top panel****: Western blot of ESRP1 in PC3 control and ESRP1 overexpressing cells. LNCaP was used as a positive control. The blot is probed for ESRP1.* ***Middle panel****: Western blot of ESRP2 in PC3 control and ESRP2 overexpressing cells.* ***Bottom panel:*** *Western blot of ESRP1 (left) and ESRP2(right) in PC3 control and ESRP1/2 overexpressing cells. PC3 ESRP2 cells were cultured in medium supplemented with a set of different concentrations of doxycycline for 48 hours prior to protein extraction to induce ESRP2 expression.*

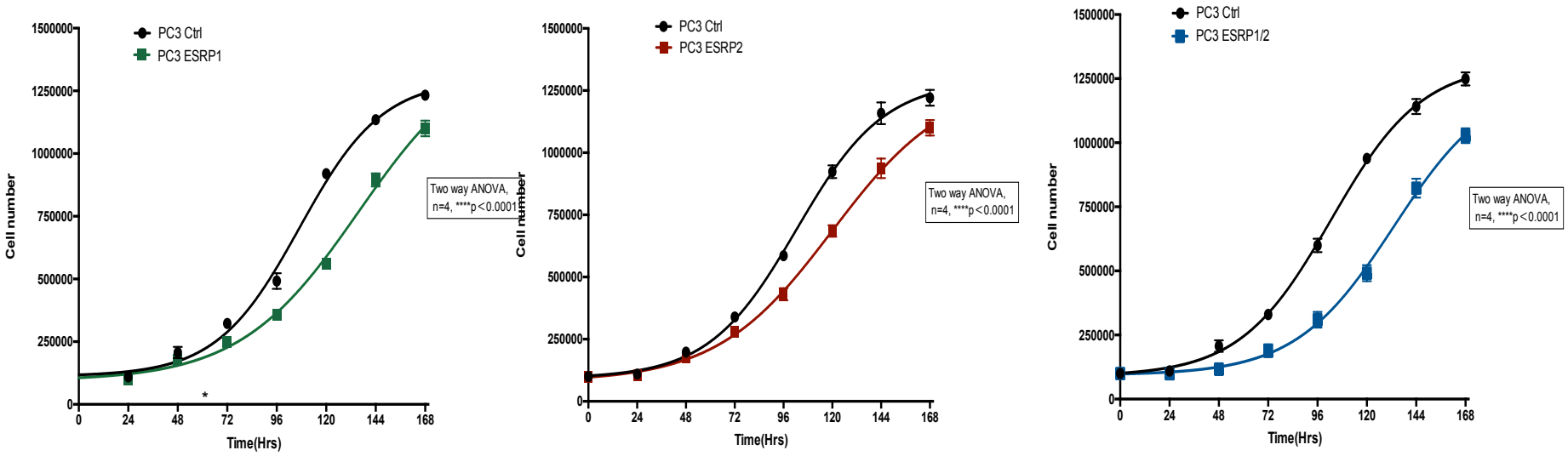


Figure 3-2 ESRPs decrease PC3 cell growth.

Left panel: *Growth curve of PC3 ESRP1 overexpressing and control cells.* ***Middle panel:*** *Growth curve of PC3 ESRP2 overexpressing and control cells.* ***Right panel:*** *Growth curve of PC3 ESRP1/2 double overexpressing and control cells. All the three cell lines – PC3 overexpressing ESRP1 only, ESRP2 only and both ESRP1 and 2 showed significantly decreased cell growth. PC3 EV and ESRP1 cells were seeded with 100,000 cells per well in a 12-well plate using 8 plates. The medium was refreshed every 48 hours after seeding. Cells were counted every 24 hours after seeding in the plate. All data was calculated and analysed with Prism and tested by two-way ANOVA. All the treatments had 4 repeats. n=12, ***p <0.001 by one-way ANOVA.*

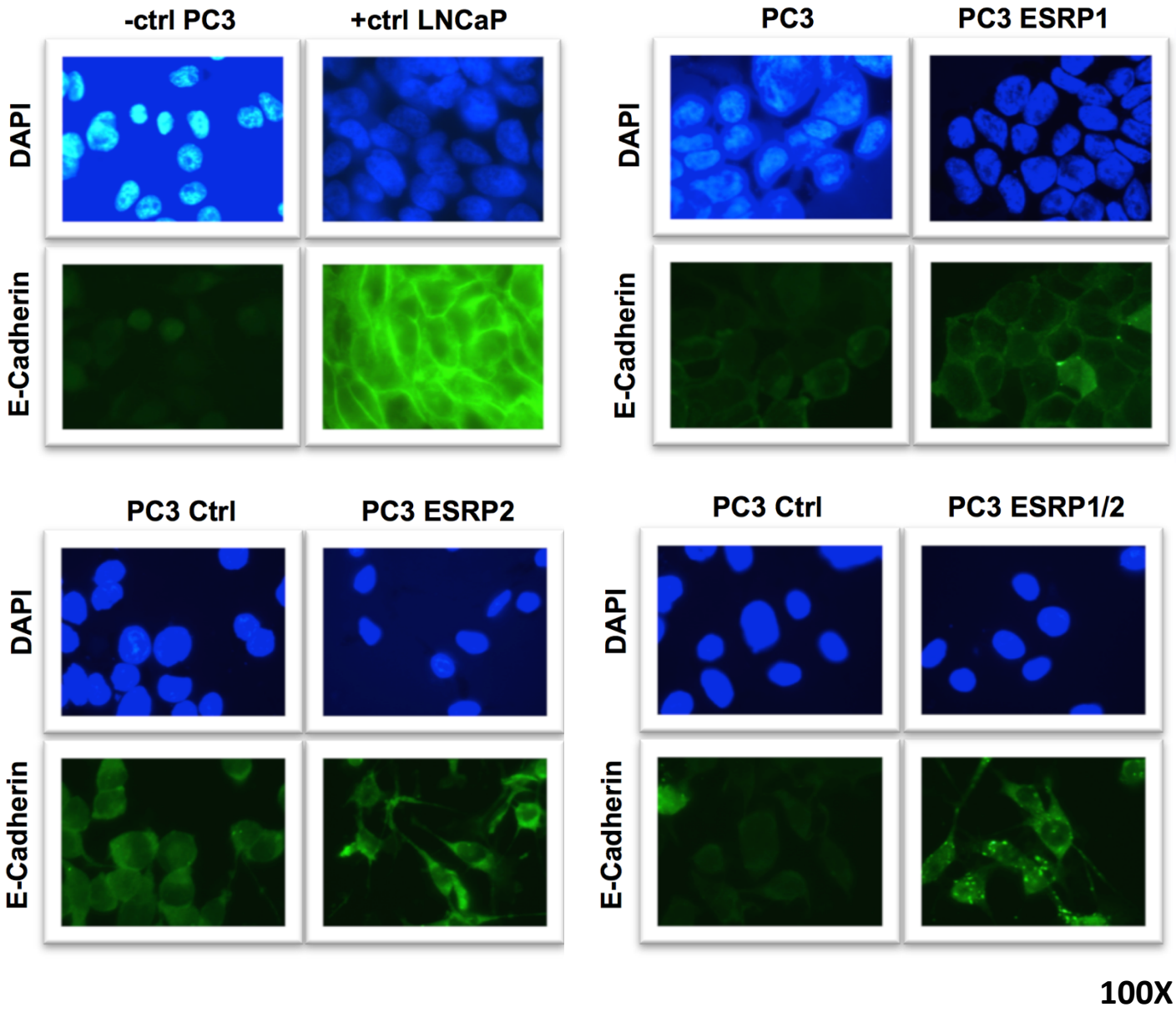


Figure 3-3 ESRPs change E-cadherin localization in PC3 cells.

Immunofluorescence analysis was carried out after transfection of ESRP1, ESRP2 and empty vector plasmid in PC3 cells. ***Upper-left panel:*** *Immunofluorescence of E-cadherin in PC3 and LNCaP cells, PC3 cells stained with mouse IgG were used as negative control and LNCaP cells were used as a positive control.* ***Upper-right panel:*** *Immunofluorescence of E-cadherin in PC3 ESRP1 overexpressing and control cells.* ***Bottom-left panel:*** *Immunofluorescence of E-cadherin in PC3 ESRP2 overexpressing and control cells.* ***Bottom-right panel:*** *Immunofluorescence of E-cadherin in PC3 ESRP1/2 double overexpressing and control cells. PC3 ESRP2 and PC3 ESRP1/2 cells were grown in a medium supplemented with 2.5µg/ml doxycycline for 48 hours prior to staining to induce ESRP2 expression.*

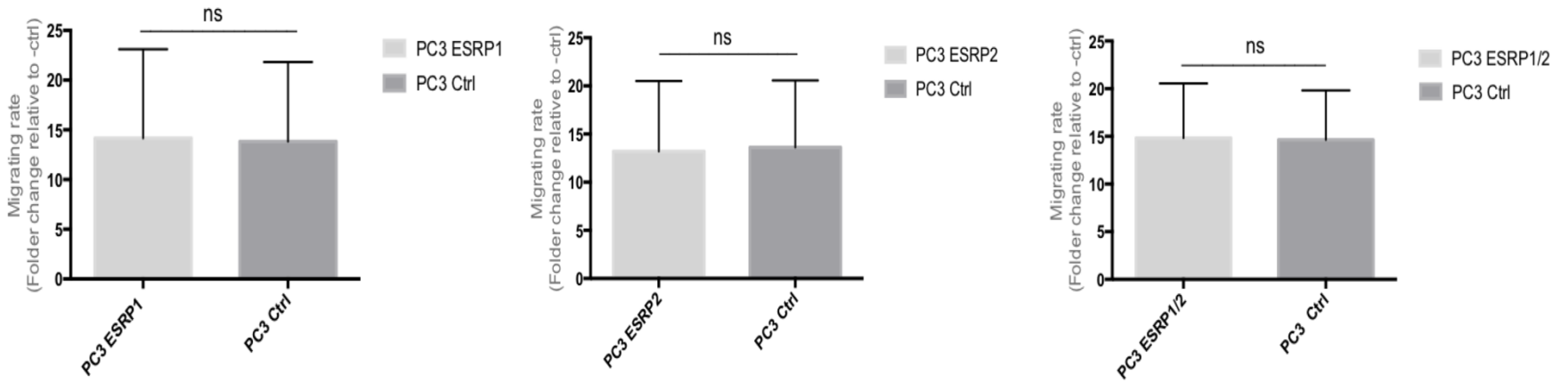


Figure 3-4 ESRPs do not affect migration rate in PC3 cells.

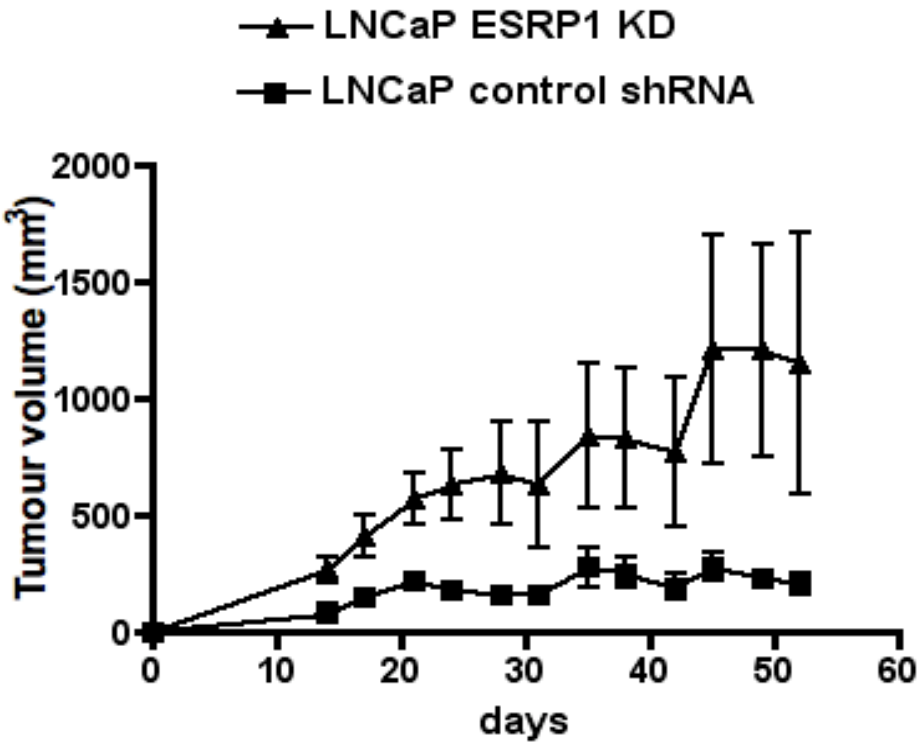
Boyden chamber assay was performed following transfection of ESRP1, ESRP2 and empty vector plasmid in PC3 cells. PC3 cells were cultured overnight in reduced medium with 2% FBS for starving cells. ***Left panel:*** *Normalized migration rate on Boyden chamber assay of PC3 ESRP1 overexpressing and control cells.* ***Middle panel:*** *Normalized migration rate on Boyden chamber assay of PC3 ESRP2 overexpressing and control cells.* ***Right panel:*** *Normalized migration rate on Boyden chamber assay of PC3 ESRP1/2 double overexpressing and control cells. PC3 ESRP2 and PC3 ESRP1/2 cells were grown in a medium supplemented with 2.5µg/ml doxycycline for 48 hours prior to experiment to induce ESRP2 expression. n=4, data was analyzed by one-way ANOVA.*

3.2.2 *The effects of ESRPs on tumour growth*

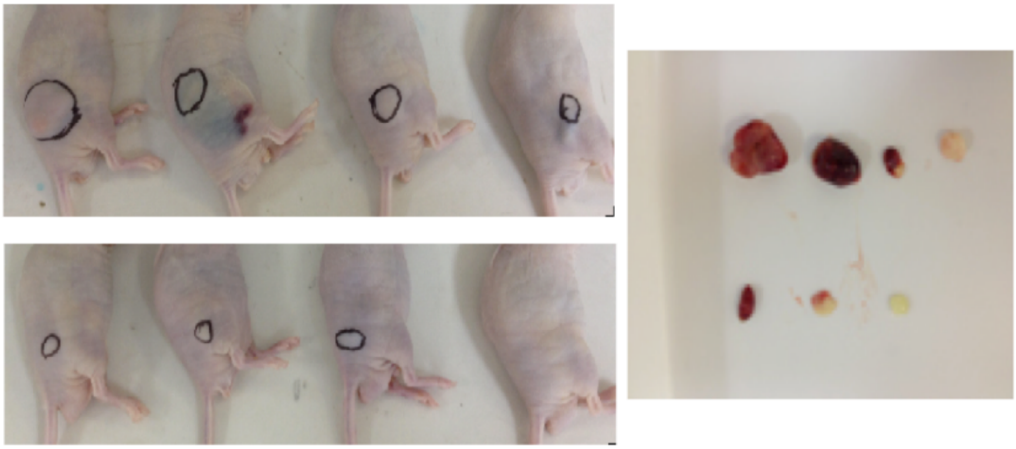
Many studies have shown that ESRPs can inhibit EMT progression in tumours, resulting in inhibition of many types of tumour growth, including colorectal tumours and breast cancers. However, there are few reports about the ESRPs' effect on the growth of prostate tumours. It was reported that ESRP1 was consistently down regulated, which is repressed by ZEB1, in prostate cancer specimens (Z. X. Lu et al., 2015). Our previous *in vitro* data showed that ESRPs are essential in the EMT process and promote EMT in the reverse direction. Moreover, we want to explore whether there is a significance to manipulate ESRPs functions *in vivo.*

To explore whether ESRP1 is important in prostate cancer tumour growth, we firstly tried to knock down ESRP1 in LNCaP cells. LNCaP is a prostate cancer cell line, which is more epithelial in phenotype and grows slowly in xenografts. According to previous *in vivo* experiments, when ESRP1 only is knockdown, there is a significant increase in tumour growth (Figure 3-5, the results were obtained by previous student Hanna Zelinska from Oltean's group).

It will be very meaningful if we can illustrate what will happen when ESRPs are overexpressed in another more mesenchymal prostate cancer cell line – PC3 cells, which usually grow in xenografts in 2-5 weeks. One million PC3 ESRP1 overexpression or vehicle cells were injected subcutaneously in the right flank of male nude mice and tumour diameters were measured by callipers. 6 nude mice were used in each group. ESRP1 overexpression decreased PC3 tumour growth significantly (Figure 3-6).



LNCaP - ESRP1 KD Day 52



LNCaP - Ctrl shRNA Day 52

Figure 3-5 ESRP1-knockdown (KD) accelerates tumour growth in LNCaP xenografts in nude mice.

Upper panel*: Quantitation of the tumour volumes in control and ESRP1 knockdown mice.* ***Bottom panel****: Examples of tumour growth in mice and extracted tumours (tumours outlined in black). Tumour diameters were measured by calipers. n=6, data was analyzed by two-way ANOVA. The results were obtained by previous student Hanna Zelinska from Oltean's group*

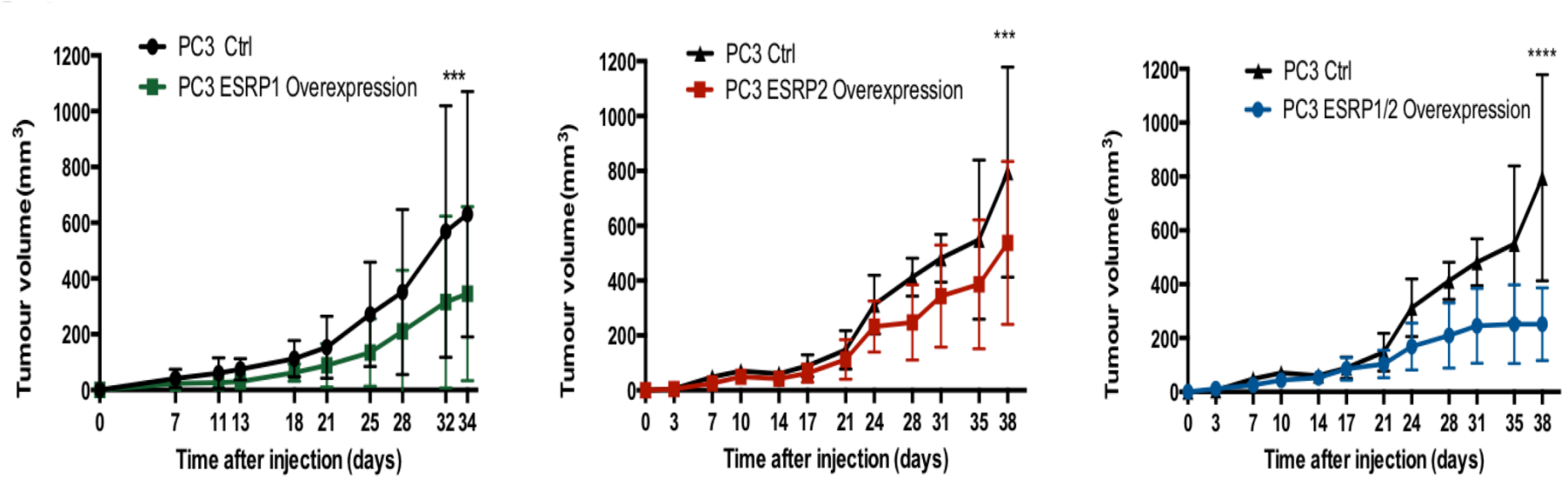


Figure 3-6 ESRPs overexpression decrease tumour growth in PC3 xenografts.

Left panel: *Quantitation of the tumour volumes in control and ESRP1 overexpressing mice.* ***Middle panel:*** *Quantitation of the tumour volumes in control and ESRP2 overexpressing mice.* ***Right panel:*** *Quantitation of the tumour volumes in control and ESRP1/2 double overexpressing mice. n=6, ****p<0.0001 by Two-way ANOVA.*

Two million PC3 ESRP2 overexpressing, ESRP1/2 double overexpressing or control (empty vector) cells were injected subcutaneously in the right flank of male nude mice and tumour diameters were measured by calipers. PC3 ESRP2 and PC3 ESRP1/2 cells were cultured in a medium supplemented with 2.5μg/ml doxycycline for 48 hours prior to injecting into nude mice to induce ESRP2 expression. Six nude mice were used in each group. Both experimental mice groups showed significant decrease in tumour growth (Figure 3-7).

All three *in vivo* experiments on ESRPs overexpressing PC3 xenografts combined with the ESRP1 knockdown *in vivo* experiment were consistent with *in vitro* experiments – suggesting that ESRPs have tumour suppressor functions**.**

According to previous reports on ESRPs mechanisms, to clarify how ESRPs affect tumour growth, we analysed E-cadherin expression in the tumour xenografts – E-cadherin is an essential biomarker of EMT in tumour growth. Total protein was extracted from tumour tissues and used for western blot on E-cadherin level. The western blot analysis showed that all the three groups overexpressing ESRPs had increase on E-cadherin expression compared to control groups (Figure 3-8).

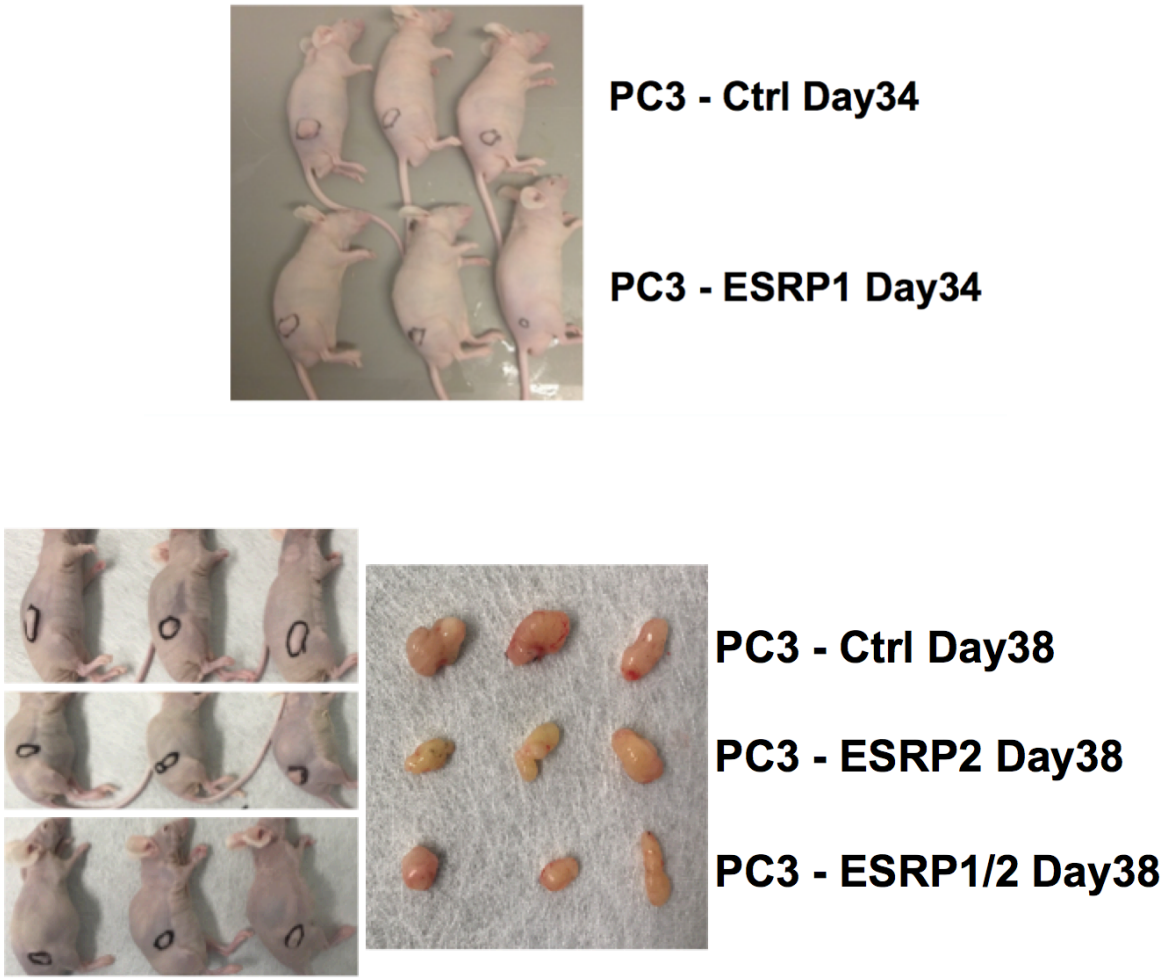


Figure 3-7 Examples of tumour growth in control and ESRP1, ESRP2 and ESRPs overexpressing mice groups.

Upper panel: *Examples of tumour growth in control and ESRP1 overexpressing mice groups.* ***Bottom panel:*** *Examples of tumour growth in control, ESRP2 overexpressing and ESRP1/2 double overexpressing mice groups (tumours outlined in black).*

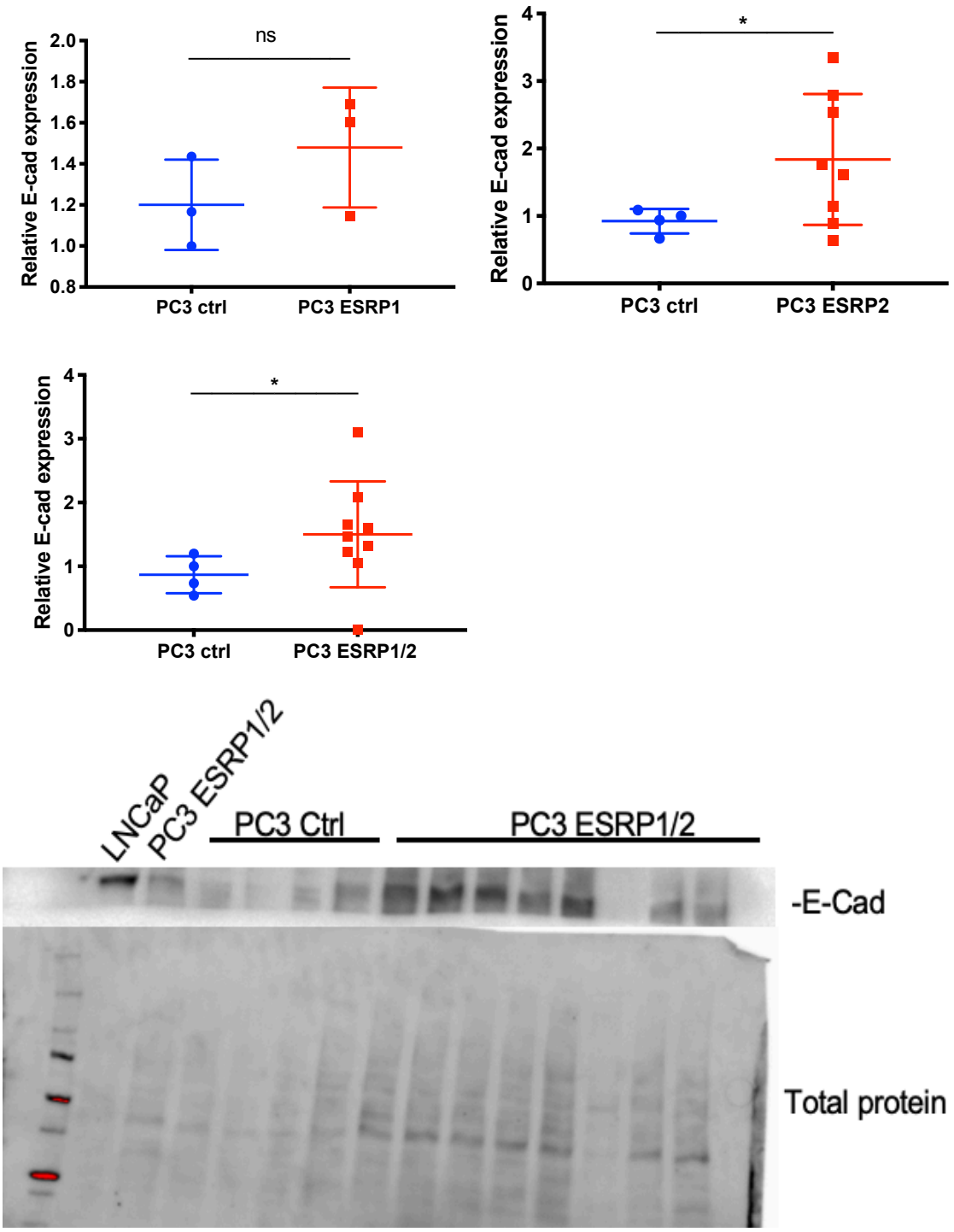


Figure 3-8 Overexpression of ESRPs promotes E-cadherin expression in tumour xenografts in vivo.

Upper panel: ***Left****: E-cadherin expression fold change in ESRP1 overexpressing PC3 vs ctrl xenograft by western blot probing on E-cadherin.* ***Right****: E-cadherin expression fold change in ESRP2 overexpressing PC3 xenograft vs ctrl by western blot probing on E-cadherin.* ***Middle panel:*** *E-cadherin expression fold change in ESRP1/2 overexpressing PC3 xenograft vs ctrl by western blot probing on E-cadherin* ***Bottom panel:*** *example of western blot probing on E-cadherin of ESRP1&2 overexpressing PC3 vs ctrl xenograft. Western blot image was analysed by Image J, and total proteins were quantitated by Image Lab. N=3-9, Data was analysed by Paired T-Test, *p<0.05.*

3.3 Discussion

3.3.1 *ESRPs have tumour suppressor functions*

In recent years there have been several publications reporting that ESRPs are low expressed in many different kinds of cancers, such as ovarian cancer, breast cancer, and colorectal cancer (Fagoonee et al., 2017; Hu, Li, Zhang, Zhuang, & Hu, 2017; Jeong et al., 2017). Based on the ESRPs effect and mechanisms in EMT, most researchers introduced a tumour suppression function of ESRPs. Many studies verified that ESRPs were important in suppressing tumour growth. However, recently several publications have shown that ESRP1 promotes human colorectal cancer progression (L. Chen et al., 2017; Fagoonee et al., 2017; Jeong et al., 2017). This triggers the question of how a gene whose overexpression is positively correlated with tumour growth determines its role in inhibiting tumour growth or promoting tumour growth. Generally speaking, there are three possibilities. Firstly, when the tumour initializes, the body starts a self-defence function that up-regulates compensatory expression of the ESRPs to try and stop tumour growth. The second possible explanation is that ESRPs are overexpressed to promote tumour growth by promoting splicing of the genes that promote cell properties associated with tumour progression, but would this overcome the switch to an epithelial phenotype? Finally, it may be more likely that ESRPs increase in late tumour development because of promoting metastasis, which needs a reversal back to epithelial phenotypes.

As shown in our *in vivo* data, ESRPs overexpressing in PC3 cells slow down the tumour growth, while ESRP1 knockdown only accelerates tumour growth in LNCaP xenografts in nude mice. The two reciprocal experiments at least prove that ESRP1 definitely has tumour suppressor functions in prostate cancer. To validate whether the ESRP2 also has tumour suppressor function, we tried to knockdown ESRP2 in LNCaP as well, but unfortunately, a massive cell death appears with transient knockdown ESRP2 in LNCaP cells, so we are not able to make a stable LNCaP ESRP2 KD cell line to carry out functional assays *in vitro* and xenografts *in vivo*. Thus, we cannot be sure whether ESRP2 are tumour suppressor or oncogene. In future, we may try to construct an inducible ESRP2 knockdown in mice xenografts to verify its functions. At least, it is worth trying to

manipulate ESRPs functions, which should be a good direction of tumour therapy targets.

Furthermore, how ESRPs work to suppress tumour growth, or even promote tumour growth, will be meaningful to explore. Once we can figure out the signalling pathways that ESRPs involved to affect tumour initiation and progression, it will be very beneficial for new cancer treatment targets.

3.3.2 *ESRPs affect prostate cancer cell functions in various ways*

It has been reported that ESRP1 and ESRP2 inhibit tumour cell motility through distinctive roles (Ishii et al., 2014). However, most publications are about their similar functions (Hayakawa et al., 2017; Warzecha, Sato, et al., 2009; Warzecha, Shen, et al., 2009). As can be realized from the above data, ESRPs have varying degrees of effect on many cellular functions of prostate cancer cell lines, such as decreasing cell growth and changing epithelial marker-E-cadherin localization. When ESRP1 and ESRP2 are both expressed, these effects are greatly increased. But we did not see any difference between ESRP1 and ESRP2 functions in PC3 cell lines. It is hard to say whether they just have very similar mechanisms in PC3 cell functions, or they get a similar functional effect via different mechanisms. This will also be an attractive topic to study in the future.

Chapter 4 Screening for small molecules that can modulate *FGFR2* alternative splicing

4.1 Introduction

As alternative splicing plays a vital role in gene regulation, there are many different methods to explore the control of AS and how the modulation of AS could be linked to diseases. To explore the splicing event of a single gene or a construct, there is one useful method, high throughput screening, which could be used for studying the effects of many siRNAs, cDNAs or chemicals on AS of a gene or construct. Through this procedure, it is possible to explore how AS is modulated and small molecules selected from the screen could potentially be used as a treatment option in clinics for splicing associated diseases.

There are a lot of successful examples using splicing-sensitive reporters siRNA, cDNA and small molecule screens to explore individual alternative splicing events (Stoilov, Lin, Damoiseaux, Nikolic, & Black, 2008; Warzecha, Sato, et al., 2009). Screening for small molecules that can regulate *FGFR2* splicing will help to reveal pathways involved in controlling splicing of *FGFR2* and how the splicing pattern is changed in tumours. These may also be possible new anti-EMT treatment targets. Using the *FGFR2* reporter we aimed to screen for small molecules that could silence *FGFR2* exon IIIc and/or promote inclusion of exon IIIb.

4.1.1 *FGFR2 is a sensor of EMT and ESRPs activities*

ESRPs are cell-type-specific splicing factors controlling the switch of epithelial isoforms during the EMT. The endogenous *FGFR2* gene, as one of the well-known genes regulated by tissue-specific AS, has two mutually exclusive and highly tissue-specific heterogeneous isoforms - *FGFR2 III b* and *FGFR2 III c* (Figure 1-14). During the EMT, the splicing of *FGFR2* is switched to mesenchymal phenotype – increasing inclusion of exon IIIc and reducing inclusion of exon IIIb.

As *FGFR2* is a sensor for ESRP activity and EMT, *FGFR2*-based splicing reporter was used to find out modulators of ESRP activity and therefore EMT.

4.1.2 *FGFR2 splicing-sensitive fluorescent reporter was designed based on endogenous FGFR2 splicing patterns*

FGFR2-based fluorescent splicing sensitive reporter was used to carry out the screen (Figure 1-16). The reporter was designed based on endogenous *FGFR2* splicing patterns (Figure 1-14). It has an artificial exon followed by exon IIIc and neighbouring introns from the *FGFR2*. Exclusion of exon IIIc results in an RFP expression and GFP out-of-frame, while retention of exon IIIc gives an EGFP signal. Mesenchymal cells include exon IIIc and give a GFP signal while epithelial cells skip exon IIIc and give an RFP signal.

4.2 Results

4.2.1 *A primary screen using the Library of Pharmacologically Active Compounds*

In HEK293 cells transfected with pRGIIIc constructs, pre-mRNA of the reporter is mainly spliced by including exon IIIc in the frame of EGFP producing EGFP protein. This mimics the alternative splicing of the endogenous *FGFR2* gene - HEK293 cells have high level expression of mesenchymal *FGFR2* isoforms – *FGFR2* IIIc. A primary screen was carried out to select compounds that can switch *FGFR2* reporter splicing.

The screen was carried out using the VICTOR X multi-label plate reader. The plate reader has a range of parameters that can be adjusted to optimize fluorescent detection, including different filters, different excitation lamp energy, and different size of the measurement aperture etc. In this screen, to avoid bleed-through between the two fluorescent channels of dsRED and EGFR protein, bandwidth ranges of excitation and emission filters should be narrow. The parameters used in fluorescent detection are listed in Table 5.

	dsRED	EGFP
Excitation filter (nm)	550	485
Emission filter (nm)	632	535
Measurement height (mm)	3 (above)	3 (above)
Measurement time (secs)	3	3
Emission aperture	Small	small
Lamp energy	30000	30000

Table 5 Fluorescent protein measurement parameters used during chemical screen.

Each of the excitation or emission filters used were of the narrow bandwidth variety, only allowing passage of light within a +/- 10nm range of the stated wavelength. dsRED was measured using excitation filter with excitation range at 550 +/- 10nm and emission filter that detects at 632 +/- 10nm. EGFP was measured using excitation filter with excitation range at 485 +/- 10nm and emission filter that detects at 535 +/- 10nm.

The LOPAC®,1280 chemical library used in the screen was manufactured by Sigma Aldrich. The “LOPAC” means “Library of Pharmacologically Active Compounds”. All the 1280 compounds of the LOPAC are used in clinics and are U.S. Food and Drug Administration (FDA) approved. This library includes inhibitors, receptor ligands, pharma-developed tools (Bioactive Small Molecule Alphabetical Index, such as GSK1210151A, LY-294,002 and JNJ-40418677), and approved drugs, affecting the majority of signalling pathways and covering all major drug target classes, including GPCRs, ion channels and kinases (Joy et al., 2014).

The primary screen was carried out to select chemicals that can switch *FGFR2* reporter alternative splicing in HEK293 cells (Figure 4-1, step 1). 10,000 HEK293 pRGIIIc cells were seeded into a 96-well plate and treated with each LOPAC chemical at 10μM concentration, in triplicate. The wells at the edge of the 96-well plate were left untreated and not used as controls to avoid the edge-effect. All the chemicals were dissolved in DMSO, so the same volume DMSO treatments were set up as controls. Cells were kept in their normal culturing incubator for 48 hours before the reporter’s splicing condition was determined by measuring the dsRED and EGFP signal in each treated well using a fluorescent plate reader. The measurements for both dsRED and EGFP were compared to control and statistically analysed using one-way ANOVA. Each treatment was compared to DMSO control using Dunnett’s post-test. As the goal is to screen compounds that can decrease the mesenchymal *FGFR2* isoform or increase the epithelial isoforms, compounds that produced an EGFP reduction or dsRED increase, or both, compared to DMSO control with a P value<0.05 were selected for the next step.

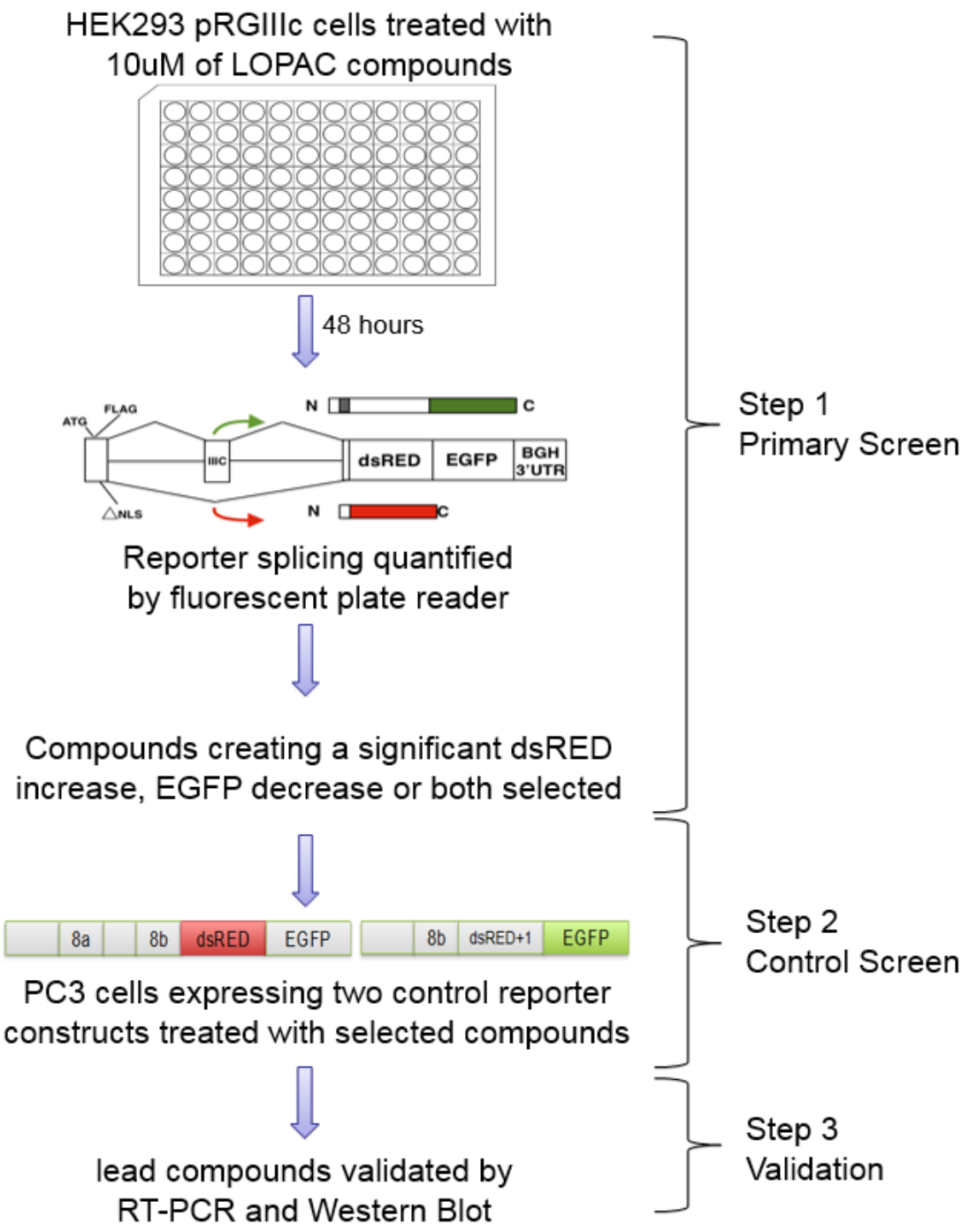


Figure 4-1 Workflow of screening for alternative splicing modulators of EMT using LOPAC library.

We have used HEK293 pRGIIIc cells to select compounds from LOPAC (Library of Pharmacologically Active Compounds) library (1280 FDA approved compounds) that affect RFP/GFP ratio by the fluorescent plate reader. And then false positives (compounds that affect either fluorescence or RNA stability) were eliminated. Finally, we have validated lead compounds using RT-PCR on endogenous FGFR2 gene and western blot on EMT markers.

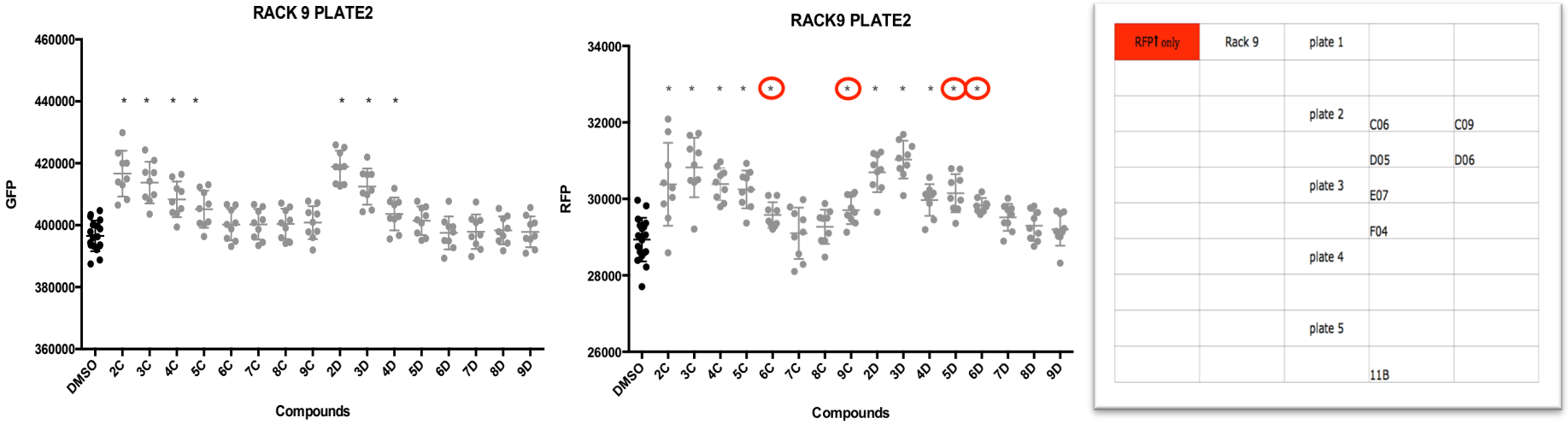


Figure 4-2 Examples for primary screen result.

Left panel *represents GFP read-outs from Vector and data analysed by Prism.* ***Middle panel*** *represents dsRED read-outs from Vector and data analysed by Prism, red circles marked the hits that increase dsRED without increasing GFP signal.* ***Right panel*** *represents the final result, in which red means RFP increase only. X-axis represents the location in the 96-well plate. Each well represents one repeat of one chemical treatment. Each treatment had three repeats. *p<0.05 by One-way ANOVA using GraphPad Prism.*

In the primary screen, there were 278 chemicals that resulted in a significant increase in dsRED or decrease in GFP or both (Figure 4-2). In principle, a switch in *FGFR2* splicing from mainly inclusion of the exon IIIc to the exon IIIb should yield an increase in dsRED and reduction in EGFP. As the predominant alterations in fluorescent protein levels were raising dsRED, some bias in the screen may occur where a small reduction in EGFP is not sufficient to be detected using this method. Therefore, chemicals that only result in raising dsRED were also picked up for the next step - control screening.

4.2.2 *Using control reporter constructs to eliminate false positive hits*

There are some other factors that affect the fluorescent protein expressing level in the reporter cells besides alternative splicing; for example, chemical treatments on reporter cells may have unknown influence on the stability of fluorescent protein or the chemicals may have fluorescence themselves. All the factors other than AS will affect the readings by fluorescent plate reader and may result in a false positive in the primary screen. To eliminate the false positive, two control reporters - PSS or DSS control reporter were used for the control screening (Figure 4-3) The control plasmid DNA did not contain the intron of the original pRG8ab (VEGF exon 8ab cloning in RG5 minigene) reporter, therefore, mimicked the mRNA transcripts without splicing events. (Figure 2-4) PC3 cells transfected with either the PSS or DSS control reporters were used for control screening.

LOPAC chemicals that produced a significant increase in dsRED or decrease in EGFP or both were selected to treat PC3 PSS and DSS control cells at 10μM for 48 hours (Figure 4-1, step 2). The control reporter mRNA cannot be spliced, therefore any changes in the fluorescence level detected by the plate reader were not because of an effect on the reporter by alternative splicing. The same as the primary screen, dsRED and EGFP readings were statistically compared to control. Chemicals that caused a significant difference ($p<0.05$) in fluorescence level were considered false positives and eliminated.

For the elimination of false-positives, 124 compounds that significantly increased the dsRED signal, or decreased EGFP or caused both were selected from 278 ‘positive’ compounds with no direct effect on fluorescent level but which may affect alternative

splicing (Figure 4-3). Following the elimination of false-positives, 26 compounds were selected.

Primary screen result		Elimination screen	Final result
Rack 10	D07	RFP⬆ only	False-positives
Rack 10	F04	RFP⬆ + GFP⬇	False-positives
Rack 13	B04	RFP⬆ only	GFP⬇ only
Rack 14	B05	RFP⬆ only	False-positives
Rack 15	E10	GFP⬇ only	RFP⬆ only

Figure 4-3 Examples of eliminating false-positive.

Red means RFP increase only, green means GFP decrease only, and yellow means both of them in the primary screen. Rack10 D07 showed RFP increase in primary screen but also showed the same result in elimination-screen, so it was eliminated. Rack13 B04 showed both changes in the primary screen while only increased RFP in the elimination screen; therefore, the effect suggested by GFP decrease may be a real one, related to splicing and not a false-positive.

4.2.3 *Validation and hit-list*

I used RT-PCR (RNA extracted from treated HEK293 cells) to check the *FGFR2* splicing at the RNA level. The HEK293 cell line is a human embryoric kidney cell line which express very clear *FGFR2* IIIc isoform.

FGFR2 has two isoforms- *FGFR2* IIIb in epithelial phenotype and *FGFR2* IIIc in mesenchymal phenotype (Figure 1-14).

Since the two exons of *FGFR2* have quite a similar size, it is difficult to distinguish only by normal PCR; therefore, restrictive digestion of the PCR products is needed to differentiate inclusion of the two different exons. *AvaI* only cuts IIIb while *Hinc*II only

cuts IIIc. *FGFR2 IIIb* isoform in a size of 243bp is cut into two fragments of 57bp and 186 bp by *AvaI*, however 240 bp isoform with IIIc is cut into 3 fragments in sizes of 63bp, 119bp and 58bp (Figure 2-1).

A mesenchymal pattern of FGFR2 restriction degest result was showed in Figure 4-4. HEK cells express clearly the mesenchymal phenotype *FGFR2* IIIc isoform, so there are no *FGFR2* IIIb cut by *AvaI*, and all *FGFR2* IIIc cut by *Hinc*II have nothing left around size 240bp. What is expected for a switch for *FGFR2* IIIb is a band uncut by *Hinc*II left around size 240bp, or bands cut by *AvaI* occurring around size 119bp, 63bp and 58bp. Chemicals show the switch for IIIb are hits that may cause an increase of ESRP activity resulting in *FGFR2* switching to the epithelial phenotype.

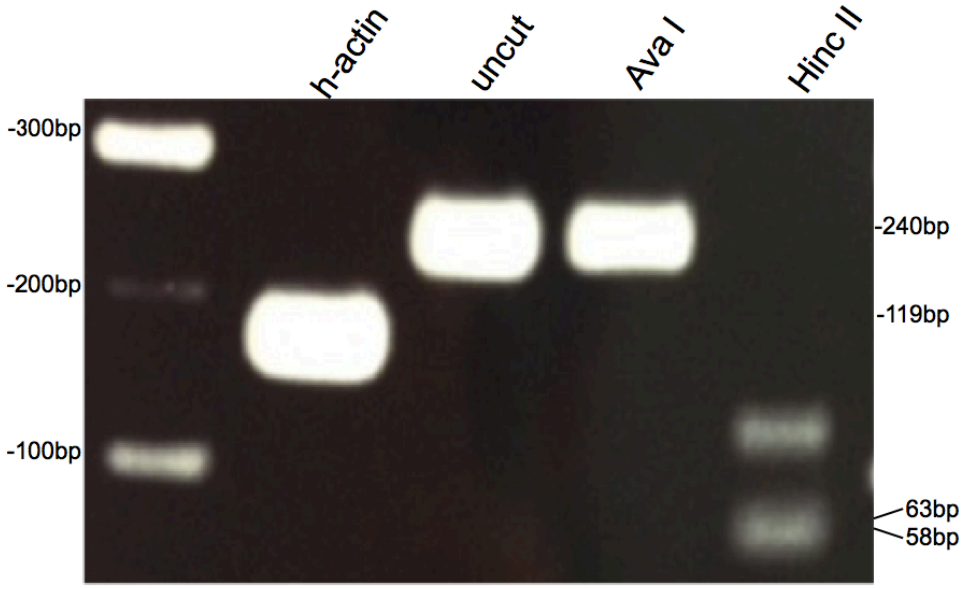


Figure 4-4 Restriction digest result of HEK293 FGFR2.

The lanes from the left represent separately h-actin control, uncut FGFR2, PCR products cut by AvaI and cut by HincII. HEK cells express clear mesenchymal phenotype FGFR2 IIIc isoform, so there are no FGFR2 IIIb cut by AvaI (no band around size 119bp, 63bp and 58bp), and all FGFR2 IIIc cut by HincII (no band left around size 240bp).

Three possible hit compounds that switch the *FGFR2* endogenous gene from isoform *FGFR2* IIIc to *FGFR2* IIIb were found in this stage. HEK cells have clear mesenchymal phenotype *FGFR2* IIIc, so for DMSO control there was no *FGFR2* IIIb cut by *Ava*I, and no *FGFR2* IIIc uncut left (Figure 4-5). For the 3 hits, bands at 240bp uncut by *Hinc*II were detected which meant the splicing program of *FGFR2* was switched to *FGFR2* IIIb.

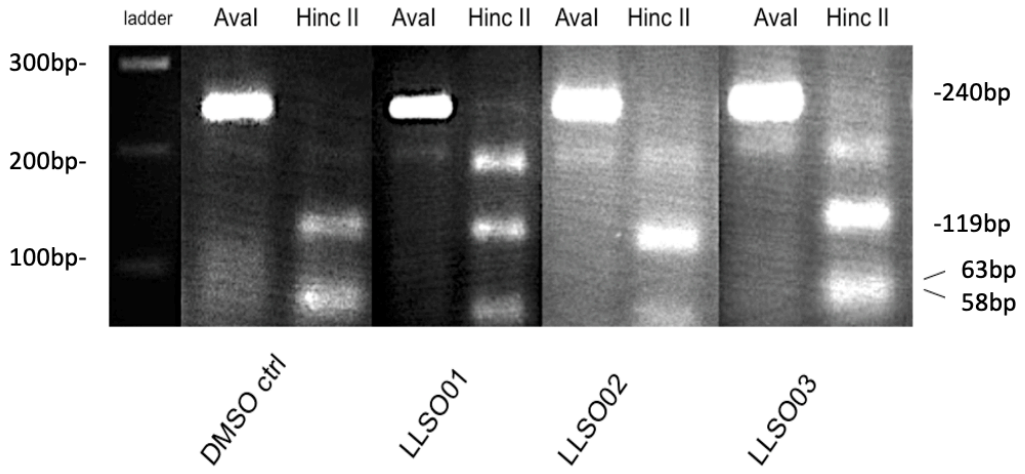


Figure 4-5 Three hit compounds change splicing of the FGFR2 endogenous gene.

Lanes from left to right represent HEK 293 cells treated by DMSO, LLSO01, LLSO02 and LLSO03; all of these compounds were used to treat cells at 10 μM for 48 hours. Lanes marked AvaI or HincII means FGFR2 PCR products cut by AvaI or HincII. DMSO control showed nothing left on 240bp for the HincII lane while all the three hits showed bands there, which means there are switches for FGFR2 IIIb under the effect of compound treatment.

4.3 Discussion

4.3.1 *Achievements and limitations of current EMT-targeted therapies*

EMT is not only related to tumour invasion, metastasis, recurrence and treatment resistance, but also stimulates tumour cells to obtain stem cell characteristics. Therefore, researchers believe that EMT, CSCs, and drug resistance constitute a deadly “triple combination” or “evil” of cancer. This "axis" (Siebzehnrubl et al., 2013; A. Singh & Settleman, 2010) has become the root cause of difficult-to-treat tumours. Therefore, targeted inhibition of EMT may play a role of “one arrow, three aims”- preventing the invasion and metastasis of tumour cells, eliminating CSCs and overcoming drug resistance (Du & Shim, 2016; Mladinich, Ruan, & Chan, 2016). It has become a highly valued new strategy for cancer treatment. (Malek, Wang, Kekoa, & Tran, 2017;

Marcucci, Stassi, & De Maria, 2016). At present, targeted suppression of EMT mainly has the following strategies:

1) Regulation of EMT-induced transcription factors

There is sufficient evidence (Lamouille et al., 2014) that EMT-inducing transcription factors play a key role in triggering EMT. EMT-induced transcription factors mainly include: SNAI1 (SNAIL), SNAI2 (SLUG), ZEB1, ZEB2, TWIST1, and TWIST2. These EMT-inducible transcription factors inhibit E-cadherin expression, leading to EMT. Therefore, inhibition of the expression and activity of these EMT-inducible transcription factors may prevent EMT from occurring (Tania et al., 2014). Studies (Hsu et al., 2013) have shown that Fucoidan can inhibit EMT of breast cancer cells by down-regulating the expression of TWIST1, SNAI1, and SNAI2.

2) Adjust the EMT signal pathway

The signalling pathways that activate EMT mainly include: transforming growth factor-β (TGF-β), FGF, EGF, phosphatidyl PI3K/Akt, Hedgehog, Notch, Wnt, MO, HGF, Insulin-like growth factor (IGF) and hypoxia/hypoxia-inducible factor signalling pathways (Gonzalez & Medici, 2014; Malek et al., 2017).

3) Targeting non-coding RNAs associated with EMT

Certain microRNAs (miRNAs) and long-chain noncoding RNAs mainly regulate EMT by regulating the expression of EMT-induced transcription factors and/or EMT signalling pathways (Diaz-Lopez, Moreno-Bueno, & Cano, 2014; Q. Xu et al., 2016; X. Zhang, Wei, Li, Liu, & Qu, 2017). Overexpression of long-chain non-coding RNA PTCSC3 inhibits EMT in glioma U87 cells by down-regulating Wnt signalling (Xia, Ji, & Zhan, 2017). Targeting EMT-associated miRNAs or long-chain non-coding RNAs has become one of the strategies for intervention in EMT.

4) Targeting EMT markers

EMT is characterized by the decrease of epithelial markers (e.g. E-cadherin, β-catenin, and cytokeratin, etc.) and the acquisition of mesenchymal markers (such as TWIST, and vimentin, etc.). Targeting these marker proteins may inhibit EMT and clear existing metastatic tumour cells. There are currently some measures targeting E-cadherin, N-

cadherin, and vimentin. Polireddy et al. screened 41,472 compounds for EMT inhibitors and found that compound BSI can upregulate E-cadherin and significantly inhibit pancreatic cancer cell migration and invasion (Polireddy et al., 2016).

At present, the research and development of drugs or compounds that target EMT inhibition is strong. There are a set of clinical trials and drug discovery experiments targeting EMT regulatory components. More than 20 types of EMT inhibitors are under study, such as salinomycin, Saracatinib, disulfiram, metformin, honokiol , and matrine (Autanski et al., 2014; Cheng & Hao, 2016; Han et al., 2015; R. Li et al., 2017; Reka et al., 2011; Santamaria, Moreno-Bueno, Portillo, & Cano, 2017; Voon, Huang, Jackson, & Thiery, 2017; J. Yang et al., 2017). Resveratrol and compounds SB-525334 and SU9516 are mostly in the preclinical stage (Grygielko et al., 2005; Lotz-Jenne et al., 2016; Santamaria et al., 2017).

In summary, EMT is a dynamic, multi-step and multi-factor regulation process. It is difficult to suppress EMT when only one process of EMT is targeted. Comprehensive targeting interventions can better prevent or reverse EMT (Malek et al., 2017). With the discovery of more specific EMT inhibitors and the combined use of some new drugs, it is expected that the treatment of tumours by targeting EMT will be achieved.

4.3.2 *Screening for regulators of FGFR2 alternative splicing*

The aim is to find small molecules that could switch *FGFR2* alternative splicing - switch the splicing event that gives mesenchymal *FGFR2* isoforms to the epithelial isoforms in this screen. The LOPAC library was a biologically annotated chemical pool of FDA approved active chemicals. The screen is aimed at drug repurposing. Drug repurposing is to inspect recognized drugs for novel usages in different disorders. Another aim is to expand our vision on the principles of disease initiation and to speed up the translation from drugs to clinical medicines. Repurposed drugs need no toxicity or safety assessment compared to probable drugs found by usual high-throughput screening (Wilkinson & Pritchard, 2015).

There are several examples of drugs used for a disease in clinics also found to be useful as anti-tumour medicines. For example, thalidomide was used to treat morning sickness, but it can cause serious neonatal defects. It is now considered an effective drug for the

treatment of leukaemia and leprosy. (Damato, Loughman, Flynn, & Folkman, 1994; Singhal et al., 1999)

There are also numerous cases of clinical drugs found by high-throughput screens to be useful for a variety of disorders including tumours. Rapamycin (sirolimus), a drug used to inhibit the mTOR signalling target by kinase, can now be used in Lymphangioleiomyomatosis (LAM) (Harari et al., 2016). Mebenozole was known as triggering toxicity in colon cancer cell lines. This drug is ordinarily used for treating gut worm infection but is currently identified as a suppressor of kinases including B-Raf as well (Nygren, Fryknas, Anagel, & Larsson, 2013). Digoxin is another example. As an anti-arrhythmic agent, Digoxin was found to decrease proliferation in prostate cancer cell lines - PC3 and LNCaP cells. A recent study by Platz et al. revealed that men who had used digoxin had a lower rate of initiating prostate cancer (Platz et al., 2011).

4.3.3 *Statistical analysis of screen data*

In large-scale high-throughput screening, many statistical approaches could be used for identifying lead chemicals from the library. Usually in the primary screening for testing a large number of chemicals, each chemical is only checked a single time for target effects. This statistical analysis that could be used to analyse the outcome is restricted. The "percentage of control" method normalizes compound activity relative to a control by dividing the raw compound measurements by the average of the controls in each plate. The measured values can then be compared between many different plates. The "Z-score" approach is typically used to choose positive hits from a big data collection. The “Z-score” has no control measurement, but it was assumed that most of the chemicals in the collection were not effective during the assay and therefore could be used as controls. So, the average value in the panel is subtracted from the measuring value of the compounds and divided by the standard deviation across the plate. These two assays both accept random distribution errors which is unmeasurable without repeating. Another factor that affects the mean and standard deviation of the entire plate is an outlier.

Since the compounds used in the screening of pRGIIIc splicing regulators are very small, it is possible to repeat the screen, accepting for computational fault and variation

analysis. The replication is determined by the accumulation and unequal spread of cells among plates of cell-based assays. Dehydration of the cell medium could also arise in edge wells of multiple-well plates, leading to different growth rate and bias. It is known that some false positives in the screen are produced by positional deviations. To minimize this, every chemical was applied in triplicate - three wells of a 96-well plate were treated. DMSO measurements of the same plate were used as the internal control when one-way AVOVA was used on the dsRED and EGFP values. Fluorescence measurements were normalized to DMSO control measurements from the same plate. Because the manual screening and measurements are not on the same day, it is better to add DMSO control to each plate rather than using the average measurements of the DMSO on all plates.

As the method I used to validate the splicing change in RNA level (generic RT-PCR and separation and visualization of products on agarose gel) is not a very sensitive method to detect tiny amounts of RNA, it is possible that some of the positive compounds are missed at this step. However, for the ones that showed change in RT-PCR, even if the changes are small at the RNA level, it may result in a significant change on the protein expression level or in functional assays.

4.3.4 *Limitations and false positive elimination within fluorescent reporter assays*

Similar to all drug discovery procedures, screening has limitations and drawbacks. Cell-based analysis is a beneficial tool in high-throughput screening processes, while the possibility of variation and noise can be enlarged by using live cells. This includes cell growth and reaction variation between different plates as well as uneven readings in the same plate. One of the common procedural problems is the "edge effect." It is well known that luminescent or fluorescent level measured from wells at the edge areas of a 96-well plate are typically much lower or higher than other areas in the plate. This may be due to the thermal gradient between the outer wells and the middle wells in the plate (Lundholt, Scudder, & Pagliaro, 2003; Malo, Hanley, Cerquozzi, Pelletier, & Nadon, 2006). This can cause uneven spreading of cells or different adhesion and morphological alterations. In LOPAC screening, cells seeded in edge wells were untreated and unused as controls to avoid "edge effects."

There were 278 'hit' chemicals selected from the primary screen that produced a significant increase in dsRED, reduction in EGFP or both. Most of these 278 chemicals had produced a significant increase in dsRED. This indicates there might be a bias when using the plate reader, that alterations in dsRED are more easily detected.

Small molecule screening has previously been used to illuminate chemicals that may alter the inclusion of an exon or the use of an alternative 5' or 3' splice site. It was reported that some screens have used splicing-sensitive reporters with luciferase or GFP output (Warzecha, Sato, et al., 2009). This kind of reporter may be useful but does have drawbacks. For instance, the effects of small molecules on the AS of reporter genes cannot always be distinguished from influences through transcription and translation. However, dual colour fluorescent splicing-sensitive reporters may potentially produce fewer false positives produced by transcription or translation as real switches in splicing should result in a rise in one fluorescent protein and a reduction in the other.

A dual colour splicing reporter system was used to screen for chemicals that can modulate the alternative splicing of microtubule-associated protein tau (MAPT) (Stoilov et al., 2008). The researchers revealed that several chemicals that changed pan gene expression also affected the ratio between the two fluorescent proteins produced from the reporter. They established that the reason for this is the RFP and GFP have different half-lives. Transcription and splicing inhibitors could also produce false positives as a result of different stabilities of the two mRNA transcripts of a reporter's AS. Furthermore, some chemicals themselves are fluorescent and fluoresces similar to the reporter fluorescent protein, which also results in a false positive.

In the *FGFR2* splicing-sensitive fluorescent reporter screen, the false positives were eliminated by using the two control reporters – PSS and DSS reporter (Figure 2-4). The control reporters do not splice as they do not have intron but exactly mimic the mature mRNA transcripts produced by alternative splicing. Any chemicals that change fluorescent protein expressing level in the primary screen through transcription or translation or via affecting RNA/protein stability, other than by splicing, will produce the same result on the control screen; while chemicals that change splicing truly will have no influence on the control reporters - no intron, no splicing.

In addition to transcriptional control and alternative splicing, there are other vital kinds of gene expression modulation that may affect reporter transcripts and eventually affect fluorescent protein expression. One such kind of mechanism is non-productive splicing, which is used to down regulate the expression of certain alternative spliced transcripts by post-transcriptional regulation with inducing NMD. NMD involves the identification of mRNA containing a premature stop codon (PTC) and prompts transcript degradation to produce a shortened protein product. Details about the NMD mechanism of detecting PTC and targeting mRNA degradation have yet to be revealed. Nonsense mutants and frameshifts can generate mRNAs containing PTC, from using alternative splice sites (Lareau, Green, Bhatnagar, & Brenner, 2004). It is indicated that 45% of human genes known to undertake alternative splicing produce one or more PTC-containing mRNAs, making them targets for NMD. This raises the concept that non-productive splicing is not only an abnormality, but regulatory machinery for post-transcriptional gene regulation (Lewis, Green, & Brenner, 2003). In addition to inducing the control of inclusion of *FGFR2* exon IIIb or IIIc, some chemicals from compound libraries can prompt the use of another sequence as a splice site in a reporter construct. These transcripts may contain PTCs that induce NMD degradation. This can serve to reduce the expression of EGFP without splicing switch to the inclusion of IIIb. The control reporter used in the screen was not spliced at the proximal or distal splice sites of VEGF because the VEGF exon 7 intron sequence has been removed. Treatment of control reporter cells may still produce products for degradation, and these chemicals could not be removed by the control screen.

Chapter 5 EMT-modulating effect of compounds identified using *FGFR2* splicing sensitive reporter

5.1 Introduction

Epithelial mesenchymal transition (EMT) indicates the transformation of epithelial cells into a biological process with interstitial phenotype cells by a specific procedure (Acloque, Adams, Fishwick, Bronner-Fraser, & Nieto, 2009; T. Chen et al., 2017; Kalluri, 2009; Kalluri & Weinberg, 2009). Its main features are reduced expression of cell adhesion proteins (such as E-cadherin) and cytoskeletal transformation (Kalluri & Weinberg, 2009). During EMT, a loss of epithelial properties like their polarization, adhesion to the BM, and acquisition of mesenchymal phenotype properties like invasion, increase of proliferation, and degradation of EM is indicated (T. Chen et al., 2017; J. M. Lee, Dedhar, Kalluri, & Thompson, 2006; Warzecha & Carstens, 2012). EMT is an extremely significant basic progression in early embryogenesis and organogenesis. It separates and migrates epithelial cells produced at specific sites from epithelial tissues to other sites and is the foundation of typical development, wound healing, and initiation of malignant epithelial tumours. EMT is key for epithelial cell-derived malignant tumour cells achieving migrating and invasion capability. To illuminate the mechanism of regulating the EMT process in tumour cells, to investigate its importance in occurrence, progression and metastasis of tumours, and to study the diagnostic techniques according to crucial factors in EMT and treatment methods pointing to EMT are the key scientific issues in the study of the EMT mechanism in tumour metastasis. Past studies have shown that various stimuli in the microenvironment can stimulate EMT in epithelial cells through a variety of different signalling pathways, both during normal development and during tumorigenesis. However, scientists have not been fully aware of the signalling pathways that prompt EMT and maintain the final status.

The PC3 cell line is isolated from human PCa bone metastasis tumours and has a low degree of differentiation. It is an androgen-independent prostate cancer cell. It does not contain endogenous androgen receptors and has moderate metastatic potential (Dillard, Lin, & Khan, 2008; Lima et al., 2018; A. L. Yang et al., 2018). For the study of

androgen-resistant prostate cancer, there is low expression of E-cadherin and high expression of Vimentin in PC3 cells. EMT is highly regulated at each stage. To investigate the effect of EMT on PC3, assays need to simulate each important stage of the process to be used to effectively evaluate potential therapeutics. In vitro assays designed to explore cell functions often focus on one phase of EMT i.e. cell growth, EMT markers, cell proliferation, migration etc (Auerbach, Lewis, Shinners, Kubai, & Akhtar, 2003; PS & JP, 2002 May). Cell proliferation can be assessed by MTT assay. The MTT assay is a colorimetric assay for measuring cell metabolic activity by measuring the absorbance of the coloured solution (mixture of cell-cultured medium and water soluble MTT) (Mosmann, 1983). Boyden chamber assays can be used to measure cell migration in response to a stimulus. In such assays, cells are cultured on a porous filter with their movement through the filter used as a quantification of their migratory phenotype (Auerbach et al., 2003; Koo et al., 2010).

In vitro EMT and cell functional assays are an important step in validating drugs or determining which genes can alter EMT. But, due to the complexity of EMT, there is no way to fully monitor EMT in cancer *in vitro*; therefore, *in vivo* models must also be employed. An example is a mouse tumour xenografts model. There are various murine models for tumour research. The main aim of these models is to clarify the mechanisms of tumour initiation, invasion and metastasis, and also to check treatment outcomes. Human tumour xenograft is most widely used among these models. In the xenograft model, human tumour cells are injected subcutaneously, in the tail vein or any other type of organ into nude mice which are immunocompromised (C. L. Morton & Houghton, 2007; J. J. Morton, Bird, Refaeli, & Jimeno, 2016; Richmond & Su, 2008). Depending on the number of cells injected, the tumour will progress around 1–8 weeks (or in some instances 1–4 months, or even longer), and the outcome of appropriate treatment administrations can be revealed *in vivo*.

In the previous chapter I described a chemical screen, which used a reporter construct to identify molecules that may influence *FGFR2* alternative splicing. Some of the chemicals were shown to increase expression of epithelial *FGFR2* isoforms. Here, I investigate whether these compounds produce a MET effect (a reversal of EMT) and which splice factors and signalling pathways mediate this effect.

5.2 Results

5.2.1 *The effects of LLSOs compounds on prostate cancer cell functions*

A library of compounds was used in a screen to identify molecules that can alter *FGFR2* alternative splicing using a splicing-sensitive fluorescent reporter that mimics *FGFR2* terminal exon splicing. This splicing decision creates either epithelial or mesenchymal *FGFR2* protein isoforms. Three compounds, with the given name LLSOs, were shown to reduce exon IIIc inclusion/ increase exon IIIb inclusion following a primary and secondary screen of a 1280 compound library. These hits had been shown to change the alternative splicing of a reporter based on *FGFR2*, increasing use of the exon IIIb involved in producing epithelial *FGFR2*. Some of the lead compounds affected the alternative splicing of endogenous *FGFR2* mRNA transcripts in HEK293 cells. Following that, I wanted to establish if any of the compounds produced a reverse EMT response. I chose to investigate this by performing several EMT and cell functional assays *in vitro* and *in vivo* including cell growth curve, EMT marker expression, cell migration assay and proliferation assays as well as *in vivo* xenograft assays.

First of all, we tried to figure out how LLSOs affect prostate cancer cell line –PC3 cells functions.

The effect of LLSOs on cell growth was investigated by cell growth curve (Figure 5-1). PC3 cells were seeded with 100,000 cells per well in a 12 well plate and cultured in RPMI1640 - media containing either control (DMSO) or one of the three drugs (LLSOs) at 10μM in 12 repeats. Cells were counted every 24 hours and the mediums with DMSO and LLSOs were changed every 48 hours. As a result, the three treatments showed different effects on PC3 cell growth: LLSO01 is toxic to PC3 cells at 10μM, LLSO02 shows no significant effect on cell growth, while LLSO03 shows a significant decrease on cell growth.

Whether or not LLSOs decrease cell migration in PC3 cells was revealed by Boyden Chamber assay (Figure 5-2). Boyden Chamber assay was performed 24 hours following seeding of different pre-treated PC3 cell lines in the inserts. Three repeats were applied for each treatment. All the four groups were pre-treated with either control (DMSO) or one of different doses of the three drugs (LLSOs) for 48 hours and then cultured

overnight in reduced medium with 2% FBS for starving cells before seeding in the inserts. PC3 cells not starved were used as negative control. All the three chemicals (LLSO01 at 5μM, LLSO02 at 10μM and LLSO03 at 10μM) significantly decrease PC3 migrating rate.

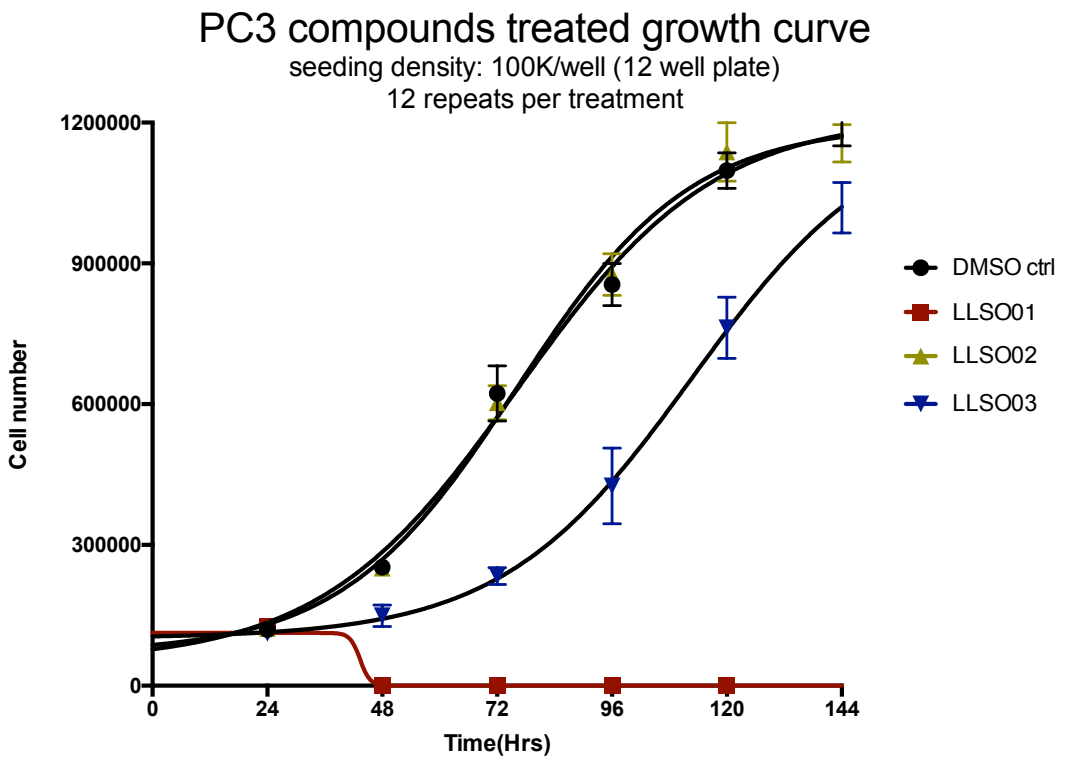


Figure 5-1 LLSOs affect PC3 cell growth in different ways.

*Growth curve of LLSOs and DMSO treated PC3 cells. For the three treatments – LLSO01(10μM) is toxic to PC3 cells, LLSO02 (10μM) shows no significant effect on cell growth, while LLSO03 (10μM) shows a significant decrease on cell growth. Medium with drugs was refreshed every 48 hours after seeding. Cells were counted every 24 hours after seeding in the plate. All the treatments had 12 repeats. n=12, ***p <0.001 by two-way ANOVA.*

Finally, whether or not LLSOs decrease cell proliferation in PC3 cells was explored by the MTT assay (Figure 5-3). All the four groups were pre-treated with either control (DMSO) or one of different doses of the three drugs (LLSOs) for 48 hours for MTT assay. Three repeats were applied for each cell line. Only LLSO01 at 5μM indicates a significant decrease absorbance rate which means a reducing proliferation rate. Additionally, LLSO01 at 1μM and 2μM also show a decrease in absorbance rate that may illustrate a dose dependent response.

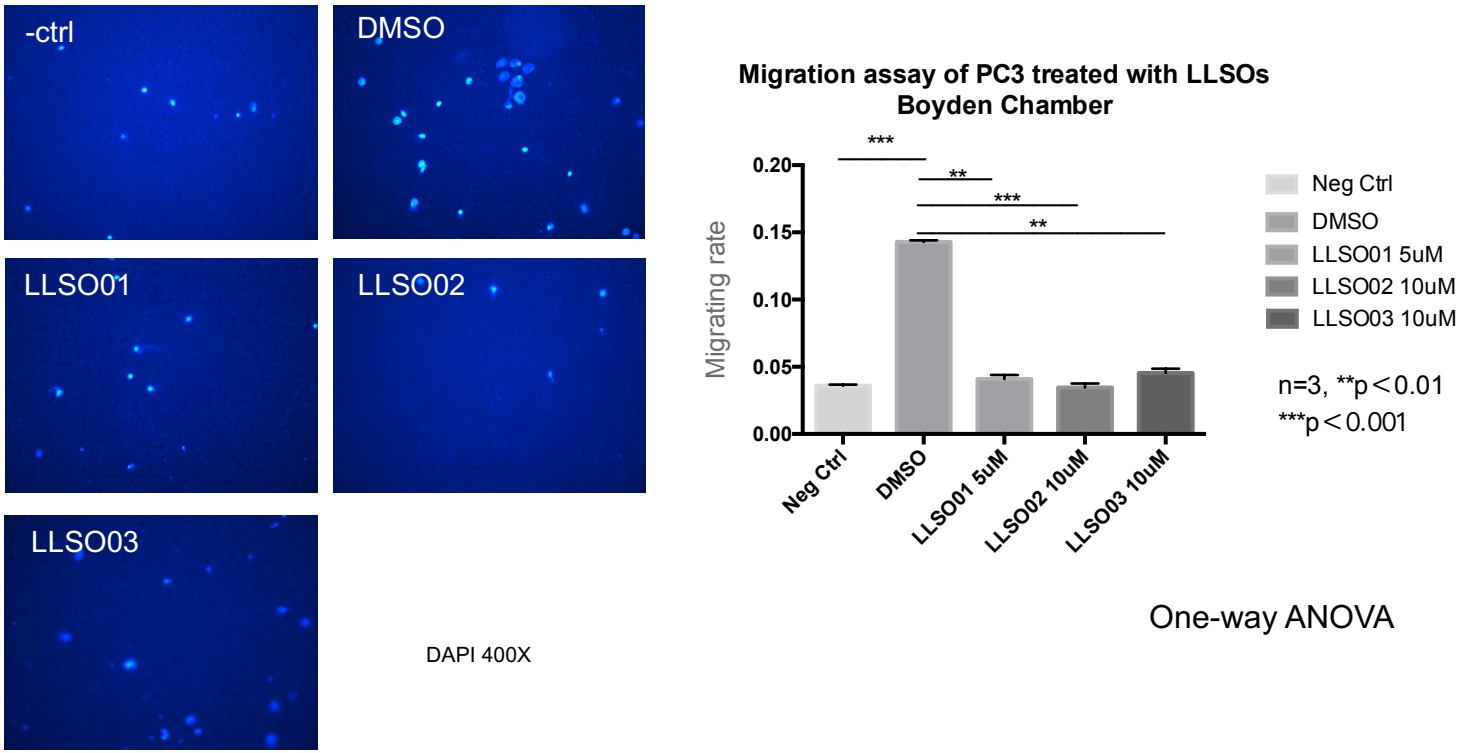


Figure 5-2 LLSOs decrease migration rate in PC3 cells.

Boyden Chamber assay was performed following 48 hours pre-treatment of DMSO and LLSOs in PC3 cells. PC3 cells were cultured overnight in reduced medium with 2% FBS for starving cells. Left panel: Normalized migration rate on Boyden chamber assay of either control (DMSO) or one of a different dose of the three drugs (LLSOs) for pre-treated PC3 cells. PC3 cells not starved were used as negative control. Right panel: Normalized migrating rate on Boyden chamber assay of LLSO01 5μM, LLSO02 10μM, LLSO03 10μM treated PC3 cells and DMSO (as control) treated PC3 cells. n=3, data was analysed by one-way ANOVA.

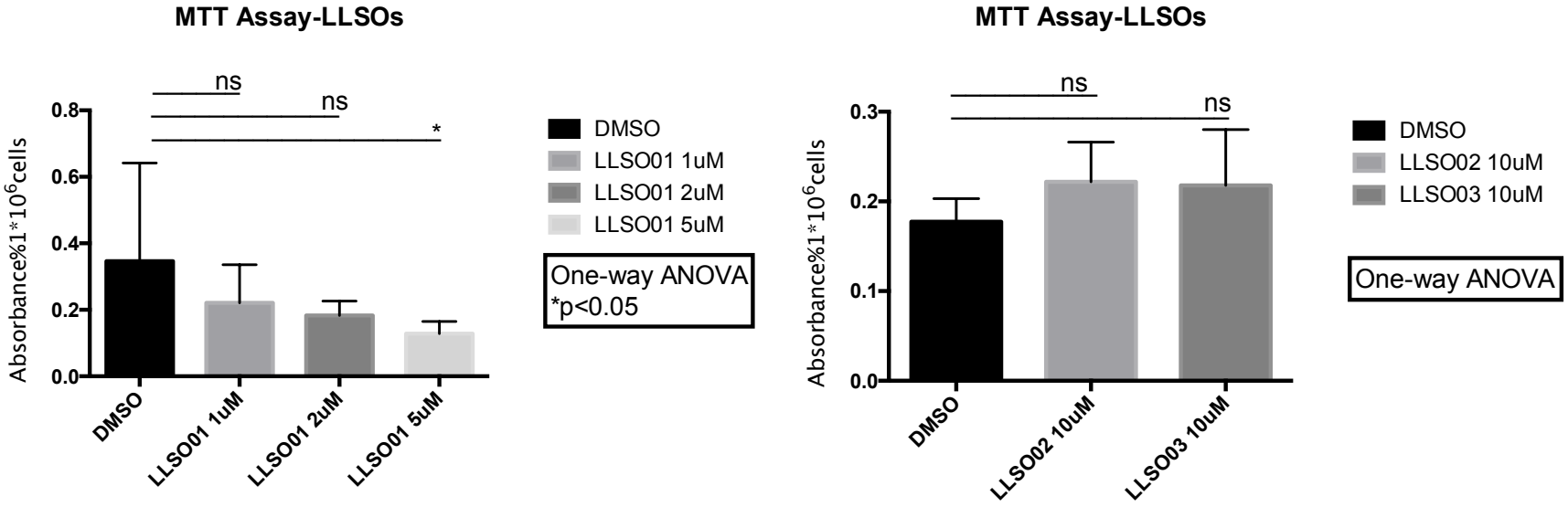


Figure 5-3 LLSOs affect proliferation rate in PC3 cells.

MTT assay was performed following 48 hours pre-treatment of DMSO and LLSOs in PC3 cells. Left panel: absorbance rate on MTT assay of PC3 treated with 1μM, 2μM and 5μM LLSO01 and DMSO (as control) PC3 cells. Right panel: absorbance rate on MTT assay of PC3 treated with LLSO02 10μM, LLSO03 10μM and DMSO (as control) PC3 cells. n=3, data was analysed by one-way ANOVA.

5.2.2 *The effects of LLSOs on EMT properties in prostate cancer cells*

Furthermore, the effect of LLSOs on EMT markers was validated by western blot and immunofluorescence analysis.

Proteins were extracted following 48 hours treatments of DMSO as control or one of the three hit compounds. Western blot of E-cadherin was performed with the LLSOs treated proteins. All the three chemicals showed increase on E-cadherin expressing level. (Figure 5-4)

PC3 cells were seeded on coverslips in a 12-well plate and treated with DMSO as control or one of the three hits for a further 48 hours. Triplicate treatments were used for control and each hit treatment. The coverslips were fixed and stained with a fluorescent antibody against E-cadherin, an epithelial cell marker. PC3 cells stained with mouse IgG instead of primary antibody were used as negative control and LNCaP cells were used as a positive control. The immunofluorescence analysis of the EMT-marker – E-cadherin established that LLSOs not only increased E-cadherin expression level, but also altered the localization in PC3 cells, making it more junctional (Figure 5-4). This result, in part, confirms that the LLSOs induce MET.

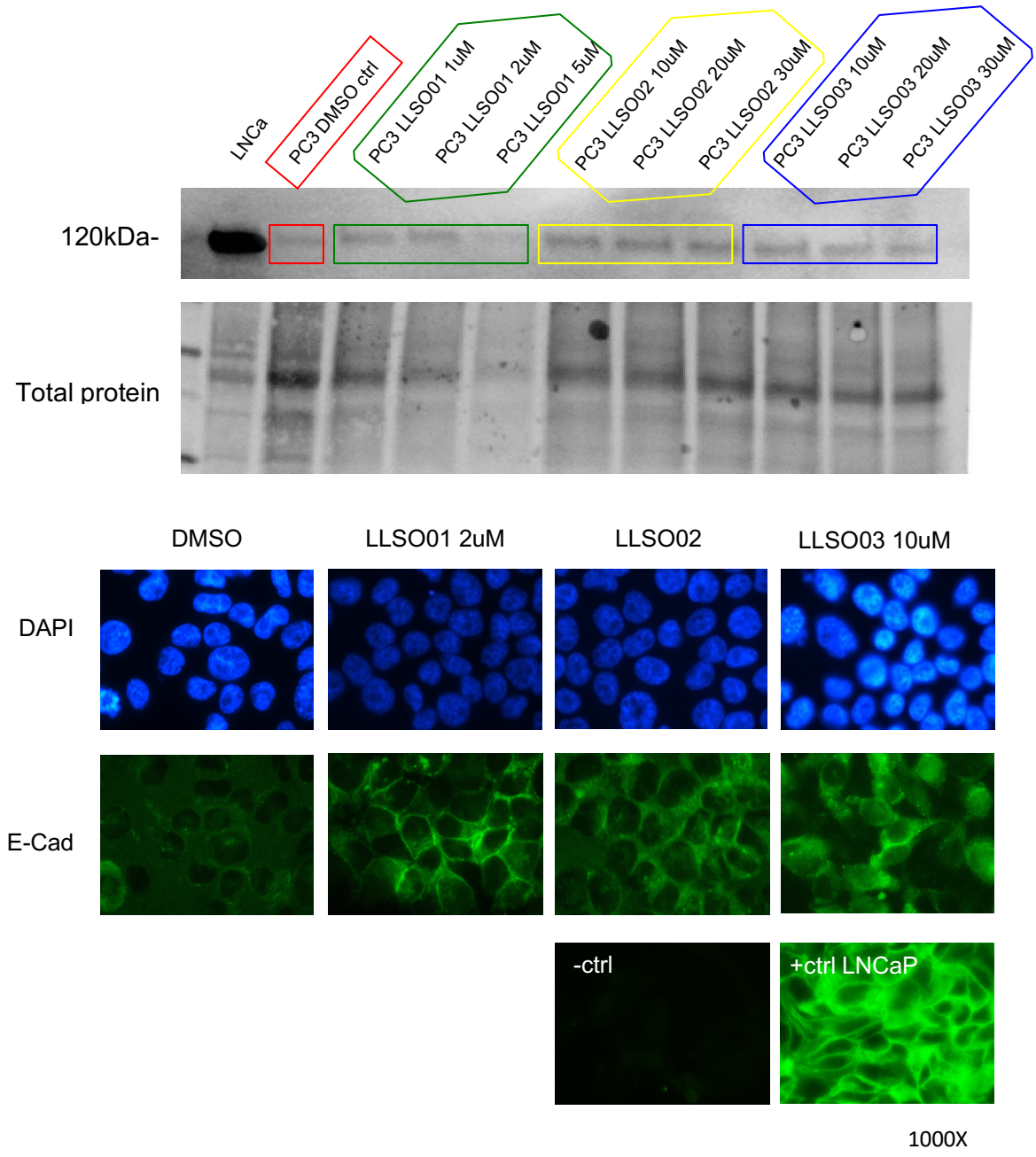


Figure 5-4 LLSOs change not only expression but also localization of E-cadherin in PC3 cells.
Top panel: *western blot of E-cadherin expression was performed with proteins extracted following 48 hours treatments with DMSO and LLSOs of different doses (LLSO1: 1μM, 2μM, 5μM; LLSO02: 10μM, 20μM, 50μM; LLSO03: 10μM,20μM, 50μM) in PC3 cells. All the three chemicals showed increase in E-cadherin expressing level.*
Bottom panel: *Immunofluorescence analysis was performed following 48 hours treatments with DMSO and LLSOs (LLSO1: 2μM; LLSO02: 10μM; LLSO03: 10μM) in PC3 cells. The staining was probed for E-cadherin. PC3 cells stained with mouse IgG were used as negative control and LNCaP cells were used as a positive control. All the three chemicals showed not only increase in E-cadherin expressing level, but also changes only on E-cadherin localization.*

5.2.3 *The effect of LLSOs compounds on tumour growth*

As my *in vitro* studies have shown that LLSOs promote MET, I wanted to see whether they are also effective *in vivo*. It will be very meaningful if we can illustrate what will happen when mice with tumours were treated with LLSOs.

To explore whether LLSOs could inhibit PCa tumour growth, we choose to use PC3 to perform xenografts. PC3 is a prostate cancer cell line, which is more mesenchymal in phenotype and generally develops in xenografts in around 2-5 weeks. From the three hit compounds, we chose to use LLSO02 as it did not have an effect on cell growth but on cell migration rate and E-cadherin expression level and localization, in this case, if LLSO02 could depress tumour xenografts growth it should be through other factors like EMT other than cell growth.

One million PC3 cells were injected subcutaneously in the right flank of male nude mice and tumour diameters were measured by callipers. Six nude mice were used in each group. Once tumour size reached 3mm by 3mm, mice were treated with either control (DMSO) or LLSO02 10μM by intraperitoneal injection twice weekly. LLSO02 significantly decreased the tumour growth (Figure 5-5).

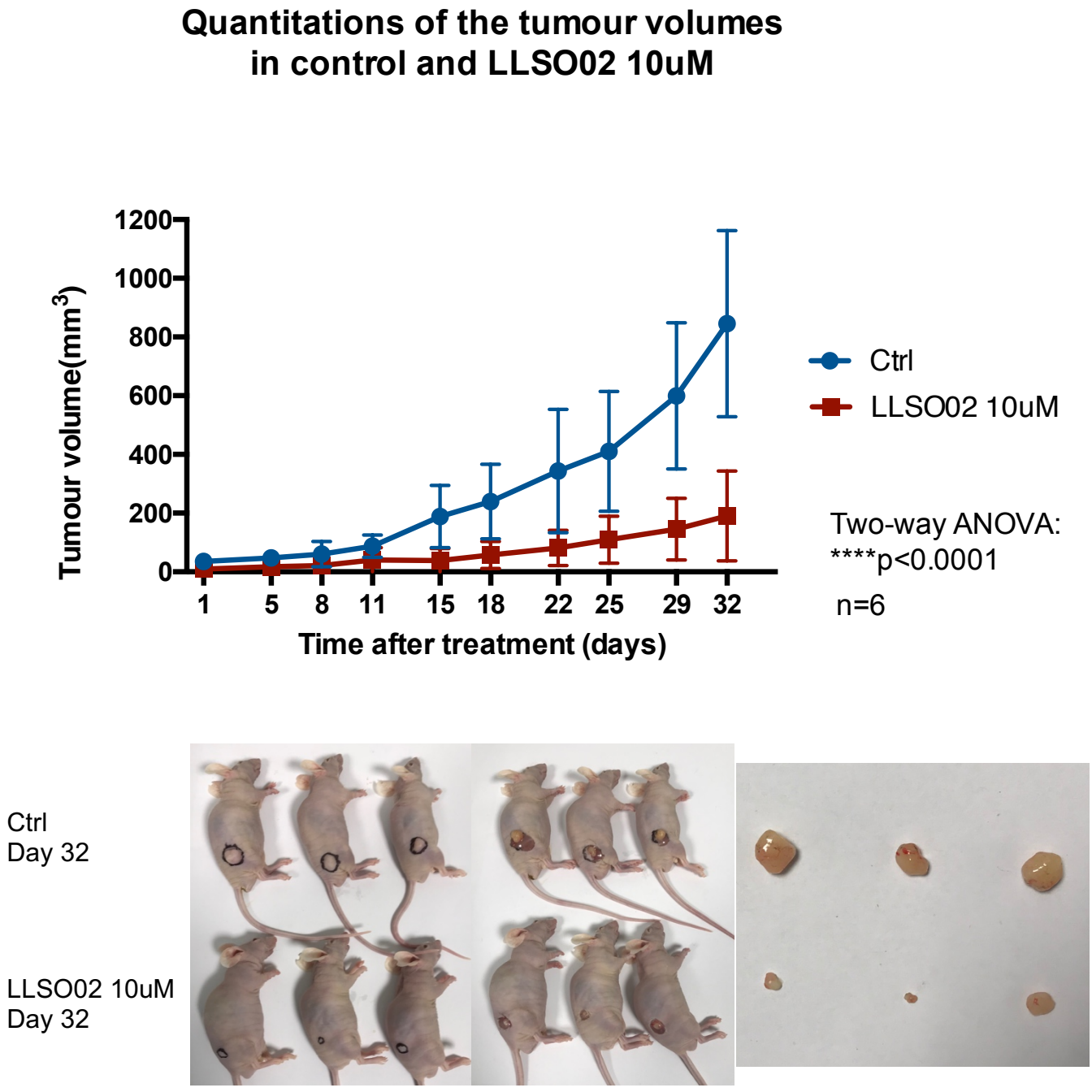


Figure 5-5 LLSO02 decreases tumour growth in PC3 xenografts in nude mice.

***Upper panel**: Quantitation of the tumour volumes in control and LLSO02 10μM treated mice.* ***Bottom panel**: Examples of tumours (tumours outlined in black; **left**: mice with tumours; **right**: extracted tumours).*

5.3 Discussion

5.3.1 *The use and limitations of EMT properties assays and cell functional assays*

To investigate EMT properties, I used E-cadherin as a marker of epithelial phenotype to perform western blotting and immunofluorescences. E-cadherin is a 120 kDa transmembrane glycoprotein of the calcium-dependent adhesion molecules family which is encoded by the CDH1 gene in humans and is essential in a variety of biological progressions like cell growth, morphogenesis, polarity, migration and tissue integrity (Huntsman & Caldas, 1998). E-cadherin is a glycoprotein whose extracellular domain can interact with E-cadherin molecules of other neighbouring cells, thereby establishing a connection between epithelial cells. The loss of expression or loss of its function can lead to the destruction of cell junctions, which is associated with the infiltration and metastasis of tumour cells. In many tumours, E-cadherin expression is changed, and decreased expression often marks a poor prognosis. Thus, E-cadherin is a sensor of EMT and also of tumour progress. However, there are all kinds of markers that distinguish epithelial cells and mesenchymal cells, such as vimentin, N-cadherin, EpCAM, TWIST and cytokeratin and so on. To use only E-cadherin as a probe of monitoring EMT properties is not so convincing, so in the future a panel of marker proteins as indicators could be used.

The cell growth curve is a common technique for measuring the absolute growth number of cells; it is also an important indicator for determining cell viability and is one of the basic parameters for evaluating cell characteristics. Our growth curve was obtained by plotting the number of surviving cells (10 000/mL) against the incubation time (hours). Otherwise, there are some other methods used to draw cell growth curves, such as MTT assay which is also used for analysing cell proliferation rate, CCK-8 assay and other colorimetric methods.(M. V. Berridge & Tan, 1993; Cory, Owen, Barltrop, & Cory, 1991; Mosmann, 1983) The operation flow of MTT and CCK-8 assay is more complicated, but they can both avoid deviations in the counting process.

Motility is a vital characteristic of live cells. Cell migration plays a vital role in embryogenesis, immune system, and many disease progressions like tumour metastasis and invasion (Braeutigam et al., 2014; Koo et al., 2010; Lauffenburger & Horwitz, 1996).

Hence, it is extremely important to find a suitable method to analyse cell migration, and also in research in many relevant fields (Guy et al., 2017; Justus, Leffler, Ruiz-Echevarria, & Yang, 2014; Kramer et al., 2013). The most commercially used cell migration assay is Boyden chamber assay.(Boyden, 1962) The Boyden chamber assay is relatively simple to perform; however, it has several limitations. The assay is difficult and time consuming, has poor reproducibility, requires a large number of cells to obtain a signal, and the movement of cells through the filter cannot be visualized.(Guy et al., 2017)

5.3.2 *In vitro assays identified compounds with potential EMT modulation activity*

E-cadherin is highly expressed by various cell lines including cancer cells. The expression or absence of E-cadherin function involves the development and metastasis of cancer. Decrease of E-cadherin reduces the strength of cell adhesion in tissue, resulting in increased motility. This can in turn allow cancer cells to attack surrounding tissues through the BM. The lead compounds identified were initially tested for EMT activity by treating PC3 for 48 hours with 10μM of each small molecule before extracting the total proteins and performing western blots to probe E-cadherin. Furthermore, immunofluorescences probing E-cadherin was also performed following treating PC3 on coverslips in a 12-well plate for 48 hours with 2uM, 5uM, or 10μM of each small molecule. Three of the small molecules, NNC 55-0396 hydrate (LLSO01), Nemadipine-A (LLSO02), and Naltrexone hydrochloride (LLSO03) significantly increased the E-cadherin expressing level and enhanced cell-cell junction (Figure 5-4). This indicates these compounds can produce an effect on prostate cancer cells improving their adhesive ability; whereas *in vivo*, cell-cell junctions are usually preventing tumour cells from invading the surrounding tissue through the BM.

5.3.3 *What are the connections between LLSOs, EMT properties and tumour growth?*

NNC 55-0396 hydrate which is toxic to PC3 at 10uM, is a selective T-type calcium channel inhibitor, while Nemadipine-A is a cell permeable L-type calcium channel alpha1-subunit antagonist. It was revealed that TGF-β-induced EMT in human breast cancer cells is correlated with modifications in endoplasmic reticulum calcium homeostasis (Mahdi, Cheng, Li, & Feng, 2015). Davis FM et al. established an essential

function of calcium signalling pathway in the initiation of EMT in human breast cancer cells. Their study shows that TRPM7 regulates EMT partly in breast cancer cells, while there are some other calcium-permeable ion channels contributing to promote calcium-dependent EMT (Davis et al., 2014). Calcium channels play a vital role in numerous biological procedures, e.g. cell growth, differentiation, and cell death (M. J. Berridge, 2012; Loughlin, 2014; Shapovalov, Skryma, & Prevarskaya, 2013). Calcium channel blockers were reported to have effects on cancer invasion. Channel proteins were recognised as modulators and may play a role in tumour progression (Shapovalov et al., 2013). Maria Mancini and Alex Toker investigated the nuclear factor of activated T cells (NFAT), one of the vital steps in the calcium channel signalling pathway in cancer. It plays a role in multiple functions during carcinoma progression including cell growth, survival, invasion and angiogenesis (Mancini & Toker, 2009). All of these findings may explain to some extent why LLSO02 could affect EMT properties and therefore tumour growth. (Figure 5-5). It might be a novel cancer therapy to inhibit metastases through modulating the calcium signalling pathway in inducing EMT in tumours.

Naltrexone hydrochloride (a competitive antagonist for μ, κ, δ, and σ-opioid receptors) is a substitute for naloxone which works better and longer through oral administration. Opioid receptors have aimed to release pain and related diseases over a long history and are still used commonly as anaesthetics in hospital (Al-Hasani & Bruchas, 2011). However, there are only a few publications that characterize the relationship between opioid receptor and EMT. Lennon FE et al. revealed that the Mu opioid receptor stimulates opioid and cell growth, immigration and EMT in human lung cancer provoked by GFs (Lennon et al., 2014). Simultaneous activation of MOR and SSTR2 influences the metastatic potential of PDAC cells, in which it was reported that a strong correlation may exist between aberrant ERK1/2 activation and a process that is important to initiating EMT (Jorand et al., 2016). There is a need to demonstrate whether opioid receptors are involved in EMT and therefore PCa progression.

Chapter 6 Discussion and the future

6.1 Alternative splicing dysregulation in disease and the potential for therapeutic intervention

The human genome sequencing work has been completed in 2005, revealing that the number of genes contained in the human genome is far less than previously estimated and much lower than the number of proteins in the cell. How is the asymmetry between the number of genes and protein species in the human body achieved? Currently, known methods for increasing protein diversity include DNA recombination, RNA editing, and AS, but AS of mRNA is the main mechanism of diversity production of proteins.

In recent years, the rapid development of high-throughput sequencing (NGS) technology, through the direct sequencing of mRNA fragments, has been able to cover most of the mRNA sequences. It has been possible to identify new subtypes resulting from splicing and accurately give quantitative information (E. T. Wang et al., 2008). The application of NGS technology has greatly promoted the understanding of alternative splicing. New data show that about 95% of human genes can be alternatively spliced, and according to the latest human genome annotation information (GENCODE Human Annotation, the data provided by Version 28, http://www.gencodegenes.org), the number of transcripts encoded on average by one gene has reached 4.14, which indicates the great contribution of alternative splicing to protein diversity.

Basic research is ultimately required to serve clinical applications. Since the era of group science, the development of sequencing technology has enabled people to quickly and accurately compare the differences in alternative splicing patterns between normal physiological conditions and pathological conditions. The data indicate that many disease occurrences are associated with alternative splicing abnormalities, including growth hormone deficiency (GHD) (Braunschweig et al., 2014), Parkinson's disease (Alieva et al., 2014), and spinal muscular atrophy (SMA) (Naryshkin et al., 2014). As early as 2004, people recognized that there are inextricable links between alternative splicing and various diseases, including various types of tumours (C, C, & G., 2008 Dec; J. Chen

& Weiss, 2015; Pajares et al., 2007; Venables, 2004). Since the alternative splicing that occurs *in vivo* is a complex process, it is a highly coordinated mechanism of various influencing factors, involving many regulatory elements and modifying factors. A mutation in any one of these key factors will alter the normal splicing pattern, resulting in a mutated transcript that encodes an abnormal protein. According to multiple genomic and proteomic data, crucial alterations of transcription factors, signalling molecules, membrane proteins, and secreted proteins in alternative splicing disorders, can often lead to disease. Fortunately, the alternative splicing that occurs in these key genes is not only to act as a predisposing factor for disease, but also more likely to act as a clinical diagnostic tool or therapeutic target providing new ideas for the ultimate cure of the disease (Feng, Qin, & Zhang, 2013).

Since AS is controlled by *cis*-elements and *trans*-elements, disruption of this regulatory mechanism can result in abnormal mRNA expression, leading to disease. There are two subtypes of the disorders according to their derivation: changes in *cis*-elements (e.g. changing splicing signals) and mutants in *trans* elements (e.g. changing splicing gear itself) (Sune-Pou et al., 2017).

In addition to changes in the splicing factor that can cause abnormalities in splicing, similarly, changes in the sequence of splice sites can also lead to splicing anomalies. SNPs - single nucleotide polymorphisms - refer to mutations at a particular site in the sequence. If this mutation occurs at the alternative splice site, it will cause splicing anomalies so that introns cannot be spliced or exons that should not be spliced are spliced out.

There are many examples of abnormal splicing resulting from mutations leading to diseases. Familial dysautonomia (FD; also known as "Riley-Day syndrome"), an Ashkenazi Jewish disorder, is a rare but well recognised set of multiple autonomic dysfunctions. (Slaugenhaupt et al., 2001) It mainly occurs in Jewish families and other racial children in Eastern Europe, and the genetic carriers of the patients' relatives are about 1/50. In 99.5% of patients with familial dysautonomia the 5' splice site of exon 20 in the IKBKAP gene is mutated by a T to C substitution located at position 6 of the 20th intron. This point mutation interrupts the pairing with U1-snRNA, which results in the u1-snRNA paring with the last 3 nucleotides of the upstream exon, and the 6

downstream nucleotides. Such mutations result in abnormal splicing of introns, leading to familial dysautonomia.

Nek2 and its splice isoform, Nek2A, is a centrosome-associated kinase that is essential for the centrosome to properly replicate, isolate, mature, and establish a bipolar spindle. A team of researchers confirmed the differences in Nek2A protein and mRNA expression of Nek2 and its splice isoforms in different stages of breast cancer and said that this could provide new indicators for the clinical diagnosis of breast cancer, and Nek2 and its splice isoforms Nek2A may act as a potential new target for breast cancer management (S. L. Wang et al., 2012). The alternative splice Nek2C of the Nek2 gene is also considered to be closely associated with the progression of breast cancer. The inhibition of Nek2C expression has proved to be an operational means to potentially target breast cancer (Liu et al., 2012).

Another example is the SMN2 gene. The SMN2 gene is important in relation to SMA disease (muscular dystrophy). SMN is a widely expressed protein that is essential in the functional diversity of eukaryotic cells. SMA is a genetic disease caused by a mutation or deletion of the SMN1 gene. Humans have two SMN genes (the gene product is necessary for the production of core snRNP complexes in cells), SMN1 and SMN2, respectively, and both can encode proteins. SMN2 is a homologue of SMN1 because selective cleavage shortens the transcript and produces few functional SMN proteins. However, in patients with SMA, the SMN1 gene is deleted, but SMN2 is retained. Studies through chemical screening and optimization showed that continuous treatment with SMN splicing regulators can selectively alter the cleavage of SMN2 pre-mRNA to produce stable, full-length SMN proteins. Additionally, these small molecules were shown to be specific to SMN genes - by performing RNA sequence analysis; these modifiers altered the expression of very few genes. SMN splicing modifiers can also prolong the life span of SMA mice, restore their normal weight, and prevent SMA from movement disorders and neuromuscular defects (Naryshkin et al., 2014).

In addition, the abnormal dynamic mechanism of the spliceosomes is also closely related to the occurrence of the disease (M. C. Wahl & Luhrmann, 2015). Spliceosomes are 60S multi-component complexes of RNA and protein molecules. This machine can perform pre-mRNA splicing by deleting introns and linking exon sequences to mature

mRNA. This is one of the important links in gene expression and regulation. From the point of view of the mechanism of action, the spliceosome, as a dynamic molecular machine, needs to be gradually assembled from the subunits to complete each splicing event.

Understanding the mechanism of action of the spliceosome in cells is of great importance and can help us understand the mechanisms of the diseases associated with these spliceosomes. By understanding this process, researchers may eventually be able to develop disease treatment strategies that fix the wrong splicing process.

For example, the spliceosome is important for hematopoietic malignancies, such as leukaemia. AML1 gene structural abnormalities are common in acute leukaemia, including balanced translocations and mutations such as point mutations, insertions or deletion mutations, resulting in the loss of transcriptional activity of AML1. AML1a is an alternative spliceosome of AML1 in which the transcript product only contains a DNA binding domain and lacks a transcriptional activation region, which results in loss of normal transcription factor function. However, this transcript product has a stronger target gene affinity than intact AML1 product, which enables it to competitively combine with the target gene and interfere with the function of the entire AML1.

Designing treatments at the level of alternative splicing has unparalleled advantages. At present, at the level of gene expression, RNA interference (RNAi) and antisense oligonucleotides are two classical treatment methods. Through the specific binding of single-stranded RNA to target mRNA, the target mRNA can be degraded to down-regulate gene expression. For example, in pancreatic cancer, abnormal pre-mRNA splicing can be detected. Serine-arginine protein kinase 1 (SRPK1) is overexpressed in pancreatic cancer cell lines, and SRPK1 is one of the major controlling proteins of pre-mRNA splicing. SRPK1 siRNA (small interfering RNA) was used to downregulate the presentation of SRPK1 and was found to cause cell growth and increase apoptosis, and even increase sensitivity of the chemotherapy drugs gemcitabine and cisplatin (Hayes, Carrigan, Beck, & Miller, 2006).

In contrast, steric-blocking oligos that have just emerged in recent years are new methods for the management of disease at the level of pre-mRNA splicing (Kole, Krainer,

& Altman, 2012; R. K. Singh & Cooper, 2012). Steric-blocking oligonucleotides can alter the pre-mRNA splicing pattern without degrading the target mRNA, hinder the splicing of the spliced complex to false exons in the spatial structure, and at the same time promote the generation of the correct subtype; ideally, it can even restore normal protein expression. (Gamazon & Stranger, 2014; Kole et al., 2012). There have been some successful cases using this strategy. For example, the combination of a specific ligand and a receptor for advanced glycation end-products can activate the relevant inflammatory signalling pathways, which cause degenerative lesions or induce hepatic tumourigenesis (Lertwittayapon, Tencomnao, & Santiyanont, 2012). The soluble RAGE subtype RAGEv1 present in the peripheral blood can neutralize these specific ligands and inhibit pro-inflammatory signalling pathways activation. By shifting the splicing of the RAGE, which makes it more likely to express soluble RAGEv1, a significant therapeutic effect can be achieved, and it is likely to become a new strategy for the treatment of liver tumours. Thus, compared to the previous two treatment approaches, the steric-block oligonucleotides can withstand more chemical modifications with good prospects in clinical application (Kole et al., 2012).

6.2 EMT in tumours and the prospective for therapeutic intervention

The EMT process is related to two important characteristics of tumours, which are tumour metastasis and tumour resistance to therapy, respectively. Both of these characteristics may be related to the stem cell-like characteristics of tumour cells resulting from EMT. Researchers have found that in mammary epithelial cells, after the expression of the TF TWIST1 or SNAI1 which induces EMT, the number of stem cell-like cells will increase significantly in cancer. This result is detected using cell surface antigen detection, gene expression profile detection, formation of mammosphere detection, and other detection methods, and the same results can be obtained with TGF-β (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Mani et al., 2008; Tran, Corsa, Biswas, Aft, & Longmore, 2011). Transcription factors that can induce EMT, such as TWIST1, FOXC2, SNAI1, ZEB2 (aka SIP1), TWIST2, etc., vimentin and fibronectin expression levels in CD44+/CD24- stem cell-like tumour cells were much higher than those at a higher degree of differentiation CD44+/CD24- in epithelioid tumour cells.

These experimental results, together with previous studies, have shown that genes associated with tumour invasion, metastasis, and angiogenesis are up-regulated in CD44+/CD24- breast cancer tumour cells, and increase cell invasion and migration. At the same time, the study also found that the number of cells exhibiting the CD44+/CD24- antigen phenotype in breast cancer cells distributed into the circulatory system and bone marrow was also significantly increased (Mani et al., 2008).

The large number of CD44+CD24-stem cell-like tumour cells and the large number of genes associated with the EMT process are associated with basal-like subtype of human breast cancer. It is revealed that proteins related to the EMT process, such as vimentin, α-smooth muscle actin, N-cadherin, SPARC protein, laminin, and fascin are abundantly expressed, while the expression of E-cadherin is reduced compared to others in breast cancer cells (Kulka et al., 2016; Scanlon, Van Tubergen, Inglehart, & D'Silva, 2013; S. S. Yang et al., 2013). Because these tumours are prone to metastasis, they have poor prognosis. The expression of FOXC2, a TF that can induce EMT, was evaluated in 117 primary invasive breast cancer samples and it was found that the factor is closely related to basal cell-like breast cancer (Hollier et al., 2013). Combined with these experimental results, the CD44 +/CD24 - phenotype increased in basal-like breast tumours.

EMT also plays a very important role in tumour resistance to treatment. In the tumour "residual" lesions following standard chemotherapy, the majority of the remaining tumour cells are stem cell-like tumour cells. For example, after neoadjuvant therapy and standard anthracycline/taxane-based chemotherapy for breast cancer patients, it can be found in the biopsy tissue that the number of CD44+/CD24- cells expressing genes related to the EMT process has increased significantly (Deng et al., 2017). However, this is not the case for ERBB2+ breast cancer patients treated with EGFR and ERBB2 dual inhibitor lapatinib (Montemurro, Valabrega, & Aglietta, 2007). For some tumours, such as lung cancer and colon cancer, the sensitivity of EGFR kinase inhibitors decreases after cells undergo EMT. This may be due to the fact that the cells activate PI3K and Akt, downstream factors of EGFR kinase, and are no longer needed in the role of the EGFR kinase signalling pathway (Buck et al., 2007; Huang & Fu, 2015; Jakobsen et al., 2016; A. F. Lee et al., 2017).

The EMT and the stem cell characteristics of tumour cells after EMT are also related to the anti-apoptotic mechanisms of tumour cells (Acloque et al., 2009). After induction of EMT by TGF-β in the mammary epithelium of EpH-4 and NMUMG mice, these epithelial cells were resistant to UV-induced apoptosis (Robson, Khaled, Abell, & Watson, 2006). Similarly, in breast cancer cells, down-regulation of let-7 miRNA expression levels can enhance the metastatic capacity of tumour cells while also making tumour cells resistant to treatment. This is because the tumour cells gain stem cell characteristics and the gene expression profile of the cell also becomes a gene expression pattern associated with the EMT process (An, Sarmiento, Tan, & Zhu, 2017; Tang, Ahmad, & Sarkar, 2012). Co-culturing tumour epithelial cells with mesenchymal fibroblasts or placing tumour epithelial cells in an oxygen-deprived environment can also make them resistant to treatment (S. W. Lee, Kwak, Kang, Park, & Jeong, 2018; Shiga et al., 2015). This may also be due to the EMT of cells.

More and more studies indicate that EMT and MET play a key role in the regulation of cellular plasticity (Jolly, Ware, Gilja, Somarelli, & Levine, 2017; Strauss, Hamerlik, Lieber, & Bartek, 2012; G. M. Wahl & Spike, 2017). At the same time, they also have an important responsibility for the treatment of tumours, metastases and recurrences, and the therapeutic tolerance of tumour cells. The role of EMT and MET programs and related regulatory factors on certain tumours, especially neuroectodermal neoplasias, mesenchymal neoplasias, and haematopoietic neoplasias also require further study. Since the results of EMT have very important clinical significance, the inhibition of the EMT has become a very promising treatment option. Nevertheless, due to the complexity of the signalling network that regulates the EMT process and the existence of a MET transformation mechanism that can reverse the EMT, the situation becomes even more complicated. In addition, we know very little regarding which tumour cells should be treated and at what stage of tumour development treatment should be given. Recent studies have shown that tumour cells have already begun systemic dissemination in early stage lesions (Kang & Pantel, 2013); in this case it is too late to give EMT suppression therapy after clinical diagnosis of tumours. However, if tumour metastasis is continuous (i.e., metastasis re-spreads and metastasizes to form the next

metastasis), the use of EMT suppression therapy may inhibit subsequent tumour metastasis.

We are not yet clear which pathways to inhibit when suppressing the EMT process to obtain the best therapeutic effect, while minimizing the toxic side effects to the body. The similarities between the EMT and the normal stem cell program make the toxic side effects of the EMT suppression therapy a major obstacle. At the same time, the reversible features of the EMT and MET also cast doubt on the efficacy of EMT suppression therapy. Another key issue is that, since tumour initiation often takes years or even decades (such as breast cancer), how can we determine whether EMT suppression is effective before tumour metastases occur?

All the problems mentioned above indicate that although researchers have made a lot of efforts to discover some of the molecular mechanisms behind EMT and MET, and also prove the clinical significance for patients with cancer, there are still a lot of important and basic issues to be addressed. However, we believe that with the unremitting efforts of researchers, these problems will be resolved one by one in the future.

6.3 Using splicing-sensitive fluorescent reporters as a screening tool

As switching of *FGFR2* alternative splicing could inactivate EMT and decrease tumour progression *in vivo (M. Katoh, 2009; Ranieri et al., 2016; Silipo, Gautrey, & Tyson-Capper, 2015; Zhao et al., 2013)*, additional study was needed to discover further small molecules that may affect *FGFR2* splicing pattern and also EMT. If such chemicals are found, and they are able to be used in cancer treatment they can help us explore how *FGFR2* splicing and therefore EMT is controlled. A splicing-sensitive fluorescent reporter was designed for this purpose to mimic the splicing event that gives either epithelial or mesenchymal *FGFR2* isoforms. Primary validation of the *FGFR2* construct and preliminary experiments suggested that the *FGFR2* splicing-sensitive fluorescent reporter may be a suitable tool for screening chemicals that may affect *FGFR2* splicing (Sebastian Oltean et al., 2008). There are a lot of examples of previous studies that have used splicing-sensitive fluorescent reporter to screen small molecules or siRNA libraries

(Arslan et al., 2013; Datta et al., 2018; Newman et al., 2006; Orengo, Bundman, & Cooper, 2006; Stoilov et al., 2008).

High-throughput or small screens typically include primary and control screens and validation in cell functional assays (Macarron et al., 2011; Malo et al., 2006; Michelini et al., 2010; Pereira & Williams, 2007). The primary screen was completed using the human embryonic kidney cell line HEK293. The EGFP and dsRED levels in cells treated with LOPAC library which contains 1,280 chemicals for repurposing were determined by a fluorescent plate reader. *FGFR2* is a sensor of EMT and ESRPs activities while EMT is a key point during initiation of cancer progression and anti-tumour treatment. The screen is to select chemicals that could switch *FGFR2* splicing and therefore EMT to decrease tumour growth and metastasis. Chemicals that raised reporter exon IIIb inclusion or decreased exon IIIc inclusion were used in the control screen that measured EGFP and dsRED without splicing event and then validated by RT-PCR on RNA level and Western blot on protein level. In the control screen, PC3 DSS (pRG8ab distal splice site control reporter) cells were used as GFP control and PC3 PSS (pRG8ab proximal splice site control reporter) cells were used as RFP control. The control plasmid DNA did not contain the intron of the original pRG8ab (VEGF exon 8ab cloning in RG5 minigene) reporter; therefore, they mimicked the mRNA transcripts that are produced when either proximal or distal reporter 3' splice site is chosen. Three compounds were validated and entered further cell functional assays.

Compared to drug screening by pharmaceutical companies, our screening scale is relatively small. Each screening technique has its benefits and disadvantages. Large libraries usually include many drug candidates that are not active, cannot penetrate cell membranes or may be toxic *in vitro* or *in vivo*, while the screen performed uses compounds that have known cellular targets in the library and are carried out using active cells instead of cell extracts or isolated proteins.

Fluorescent plate readers were used in primary screening to determine dsRED and EGFP levels in cells treated with the compounds as a measure of keeping or skipping exon IIIc. In the preliminary screening analysis, more than 200 compounds significantly raised dsRED or reduced EGFP or both. After the false positives were removed and validated, only three compounds were selected. This suggests that the fluorescent plate reader

selected many false positives and "hits" that failed verification. There are other fluorescence measuring methods that have higher screening accuracy, such as high-content screening (e.g., using an IN cell analyzer) or high-throughput flow cytometry.

6.4 Investigating the control of EMT activity by small molecules *in vitro* and *in vivo*

The aim of a screen for molecules that can modify *FGFR2* splicing was to ultimately use such molecules to inhibit EMT *in vivo*. Before animal studies are performed, there are many *in vitro* EMT activity assays that can be used (Koo et al., 2010). Each EMT activity assay has advantages and limitations. Western blot assays on EMT markers are simple to perform and reproduce but do not represent the protein properties as accurately as some other assays. Immunofluorescence assays are more accurate and could show the localization of proteins, but they are not easy to perform and hard to optimize. Boyden chamber migration assay is one of the most common migration assays and relatively simple to perform, allowing you to investigate migration with a chemotactic gradient using adherent or non-adherent cells. Meanwhile, it has a disadvantage in that usually this assay can only measure the migrating rate at the endpoint (Guy et al., 2017). The MTT assay was the first broadly established technique to analyse cell proliferation but has limitations. The absorbance detecting technique has lower sensitivity compared to fluorescent and luminescent ones to identify cell density. Moreover, it does not mimic the complexity of metastasis in the tumour microenvironment. Animal models are required to investigate if reverse-EMT activity of a novel compound inhibits tumour growth.

To explore the mechanism in tumour initiation, invasion and metastasis, one of the most commonly used models is the human tumour xenograft. Mouse subcutaneous xenografts model I used for investigating LLSO02 effects in tumours can evaluate the drug response of the tumour, provide convincing heterogeneity of tumours and allow for fast investigation of human tumour reaction to a treatment routine. However, this model only mimics a less representative tumour as mice are immunocompromised.

Several of the lead compounds identified during the *FGFR2* splicing sensitive fluorescent reporter screen exhibited reverse-EMT activity *in vitro* or *in vivo*. Some also increased *FGFR2* proximal splice site selection and increased epithelial maker E-cadherin on the

protein level. Even though *FGFR2* is known to be mainly regulated by ESRPs, it is not clearly established if the EMT activity changes observed are predominantly mediated by changes in the ESRPs expression. Whether modulation of ESRPs activities is the major mechanism that mediates the respective *in vivo* anti-tumour activity of Nemadipine-A requires further investigation.

Anti-EMT as a cancer treatment has had relatively limited success. This has been attributed to several mechanisms, including resistance to therapy and reduced delivery of chemotherapeutics to the tumour (Brabletz et al., 2018; Du & Shim, 2016; A. F. Lee et al., 2017). Small molecules that induce the expression of EMT marker E-cadherin while promoting the endogenous anti-oncogenic factor, *FGFR2* IIIb, is an attractive strategy for cancer therapy.

6.5 Potential signalling pathway of LLSOs

As the three chemicals belong to different classes of drug, their signalling pathway would be different according to their properties.

NNC-55-0396 (LLSO01) is a selective T-type calcium channel blocker which is used for the treatment of hypertension and chronic angina pectoris in clinic. As NNC-55-0396 (LLSO03) is toxic in most of the assays carried out to all the three cell lines (PC3, LNCaP and HEK293) we used, we chose first to explore the mechanism of the other two chemicals- Nemadipine-A (LLSO02) and Naltrexone hydrochloride (LLSO03).

Nemadipine-A (LLSO02) is a cell permeable L-type calcium channel alpha1-subunit antagonist, prescribed as anti-hypertension drugs. It was investigated that the nuclear factor of activated T cells (NFAT) is one of the vital steps in calcium channel signalling pathway in cancer, which play a role in multiple functions during carcinoma progression including cell growth, survival, invasion and angiogenesis (Mancini & Toker, 2009). In addition, AKT and MAPK signalling pathways are also shown to be associated with reduced apoptosis in cancer (Jin et al., 2007). According to these findings, Nemadipine-A may suppress L-type calcium channel expression, which results in an inhibition of Calmodulin signalling pathway. (Figure 6-1) Consequently, Calcineurin will be repressed and inactivate NFAT expression. NFAT could promote EMT through multiple mechanisms like suppressing E-cadherin expression. There are many genes triggered by

Nuclear NFAT, including genes coding the autotaxin and cyclooxygenase (COX-2) proteins which subsequently result in an invasion of tumour cells by a cascade of signalling pathways. NFAT could also activate gene regulation and DNA fragmentation, which promotes apoptosis and proliferation. (Mancini & Toker, 2009). Additionally, CaM signalling could also trigger CaMK activation and consequently promote CREB1 which lead to gene regulation and proliferation. Control of calcium-signalling pathways regulating EMT in cancer could potentially be a significant treatment approach for managing cancer progression.

In addition, it was validated that Nemadipine-A triggers TRAIL-induced cell death by down-regulating surviving in TRAIL-resistant cells, which means nemadipine-A is a hypothetical treatment approach for lung cancer (Park et al., 2013).

Naltrexone hydrochloride (LLSO03) is a competitive antagonist for μ, κ, δ, and σ-opioid receptors used as a supplementary treatment. Opioid receptors have aimed to treat pain and related disorders for thousands of years. Inhibitors of these receptors are still the most commonly used anaesthetics in the clinic (Al-Hasani & Bruchas, 2011).

It was reported that mu opiates, for example fentanyl and morphine, displayed dose-dependent effects on proliferation and migration in human pulmonary and dermal cells. Morphine, at doses used in the clinic, showed around 70% of the capability of VEGF to promote vessel growth and therefore promote cell migrating and growth. MNTX was reported to inhibit the mu opioid receptor (MOR) and Src, while activate tyrosine phosphatase in breast cancer cells. (Singleton, Moss, Karp, Atkins, & Janku, 2015) MOR was also demonstrated to stimulate cell migrating, cell growth and EMT induced by opioids and GFs in human lung cancer (Lennon et al., 2014). Otherwise, it was illustrated that a raised level of the MOR in human NSCLC stimulates Akt and mTOR activation, tumour progress and metastasis (Lennnon et al., 2012). According to the previous report in breast cancer and lung cancer, Naltrexone hydrochloride, as an antagonist for opioid receptors and also derivative of naltrexone like MNTX, may inhibit the MOR and result in an increase in tyrosine phosphatase activity and inhibition of Src. (Figure 6-2) Collaborating with VEGF and VEGF receptors combination, and cascade signalling, two mTOR complex (I and II) was formed consequently, one of which is needed for endothelial cell immigration and growth.

Naltrexone hydrochloride may also combine with FKBP12 and then prevent mTOR complex II construction. By acting at different levels in VEGF-signalling, mTOR inhibitors are able to act synergistically. The effect on tumour growth may be achieved by MORs through a combined effect on EMT, vessel growth, etc. Thus, the mTOR inhibitors might act through the suppression of some key proteins and steps in a VEGF-induced angiogenic signalling. However, MOR and its signalling is a potential target in tumour management if interacting with EMT, vascular growth, mTOR, Src and some other signalling pathways.

It was reported that stimulation of κ-opioid receptors (KOR) reduced gefitinib-resistant NSCLC cell growth by activating GSK3β. It suggested that the motivation of KOR may specify distinctive points for the inhibition and management of NSCLC (Kuzumaki et al., 2012). Inactivation of GSK3β promotes c-Myc and β-catenin degrading, which are upregulated in cancer. Moreover, inactive GSK3β could also inactivate the tumour suppressor p53. Furthermore, inactivation of GSK3β, which is positively correlated with the phosphorylation of GSK3β, is crucial for lung cancer development (D. Tian et al., 2006). To conclude, Naltrexone hydrochloride may work through inhibiting KOR to reduce the inactivation of cAMP, and then inhibit PKA or inactivate JNK signalling which both result in the suppression of GSK3β (Figure 6-2). GSK-3 is overexpressed in various categories of tumour, as well as prostate cancer. Most of GSK-3β is observed in high Gleason score tumours, where it could induce androgen-independent AR and/or promote other signalling, such as Akt.

These potential signalling pathways still need more work and opioid receptor inhibitor targeting Src, cAMP and JNK signals might be a potential cancer therapy strategy.

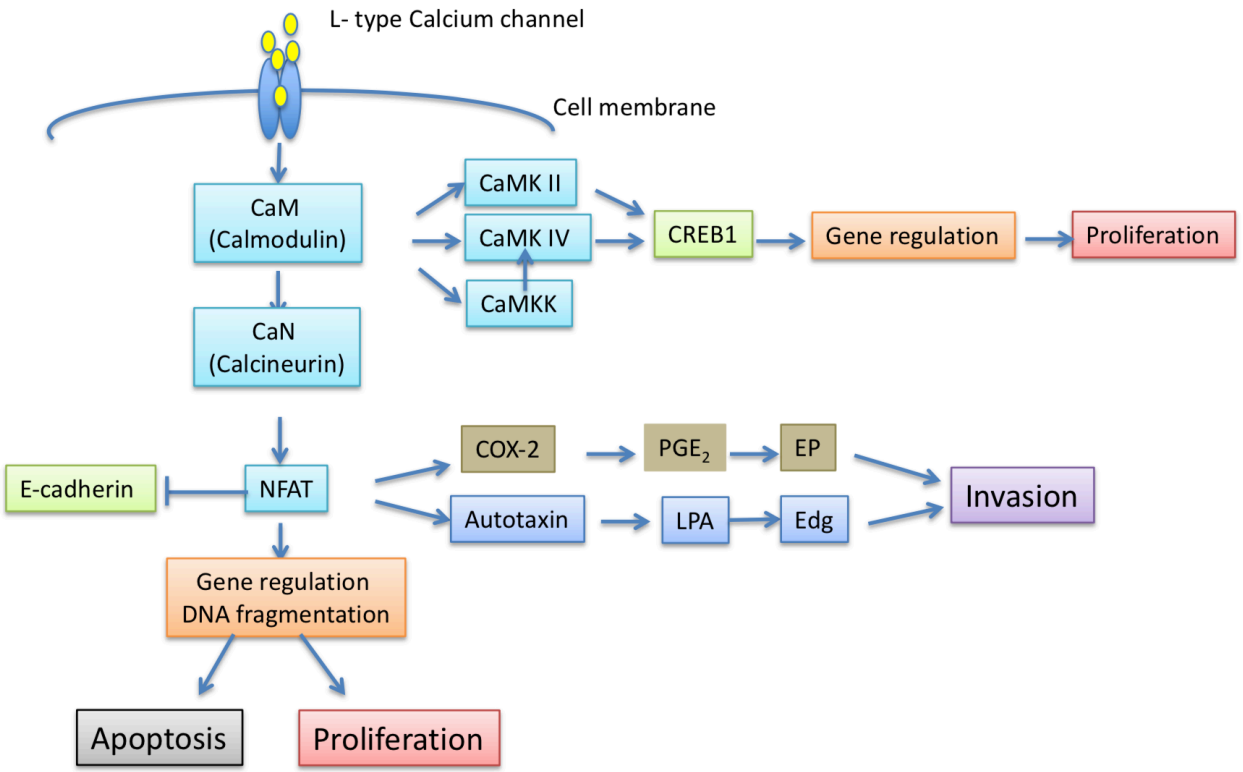


Figure 6-1 Potential Nemadipine-A signalling pathway

Nemadipine-A inhibits L-type Calcium channel which results in CaM inhibition and consequent inactivation of CaN signalling pathway. NFAT could promote EMT through multiple mechanisms including decreasing E-cadherin expression. Nuclear NFAT is involved in a complicated signalling to promote apoptosis, proliferation and invasion. In addition, CaM could activate CaMK to promote CREB1, which also results in gene regulation and then proliferation.

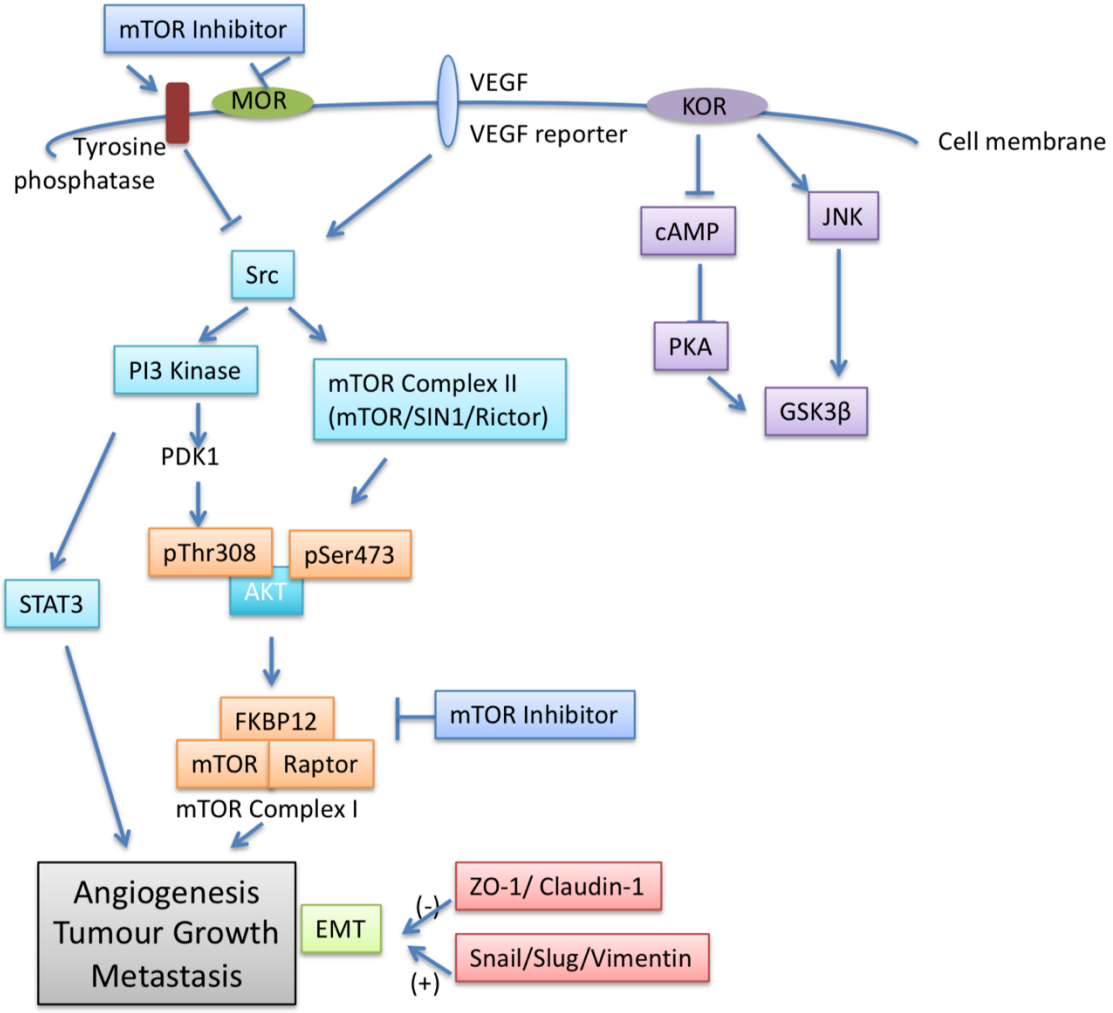


Figure 6-2 Potential Naltrexone hydrochloride signalling pathway

The combination of VEGF and VEGFRs induce Src and subsequently motivate a cascade of signalling pathways, therefore construct mTOR complex II and I that is essential for angiogenesis, tumour growth and metastasis. Naltrexone hydrochloride may inhibit the MOR and stimulate tyrosine phosphatase activity and inhibit Src. Naltrexone hydrochloride, as a mTOR inhibitor, may also bind to FKBP12 to prevent mTOR complex II construction. Naltrexone hydrochloride may also effort through cAMP/PKA/Glycogen synthase kinase (GSK-3). Adapted from (Kuzumaki et al., 2012; Lennnon et al., 2012)

Since the three chemicals were screened using *FGFR2* based fluorescent sensitive reporter, which is a sensor of EMT and ESRPs, they may affect EMT properties through splicing regulators controlling *FGFR2* splicing. Although *FGFR2* is mainly regulated by ESRPs, there are many other splice factors that may affect *FGFR2* splicing and involved in EMT (Shapiro et al., 2011; Ueda et al., 2014). EMT-regulated skipped exons were reported to be regulated by many different factors such as ESRP, PTB, HNRNPF/H, Muscleblind-like (MBNL), CUG-BP, Elav-like family (CELF) and RBFOX2, and some other unknown splice factors.(Shapiro et al., 2011). *FGFR2* exon IIIb/IIIc switch may also be regulated by those splice factors (Figure 6-3). The LLSOs potential mechanism network based on splice factors and splicing signalling pathways were indicated in Figure 6-4. There still are some unknown key points of the signalling pathway and splice factor involved in *FGFR2* splicing that need to be demonstrated. Therefore, how the splicing process connects to calcium channel and opioid receptor signalling is what needs to be explored next. Understanding LLSOs mechanisms in cancer might be a new direction for developing anti-cancer drugs.

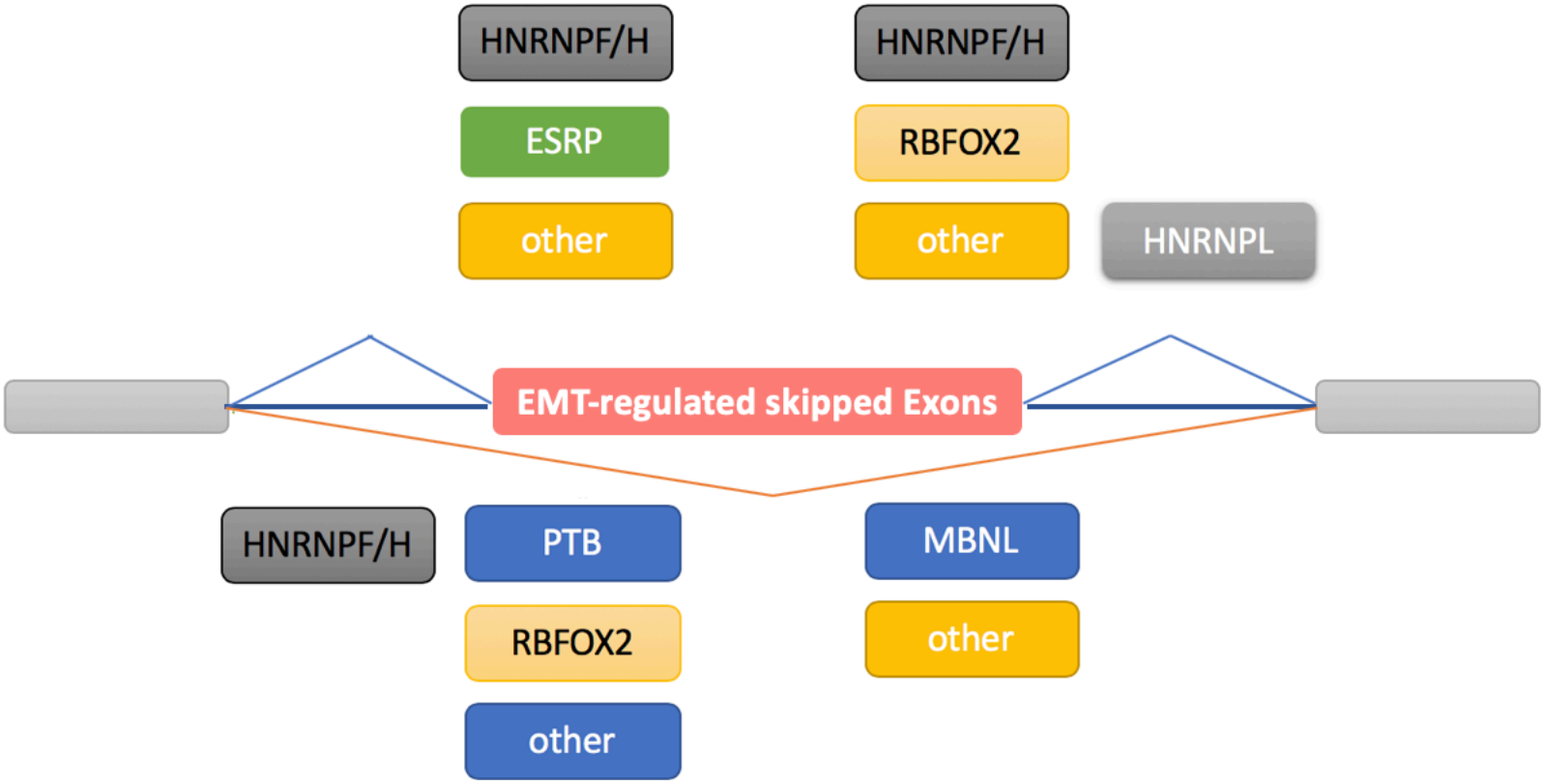


Figure 6-3 Splicing factors involved in control of EMT–specific splicing.

Like other EMT- regulated exons, FGFR2 exon IIIb and IIIc may be regulated by many other different splice factors besides ESRPs, such as PTB, HNRNPF/H, MBNL, HNRNPL, CELF and RBFOX2, and some other unknown splice factors. Adapted from (Shapiro et al., 2011)

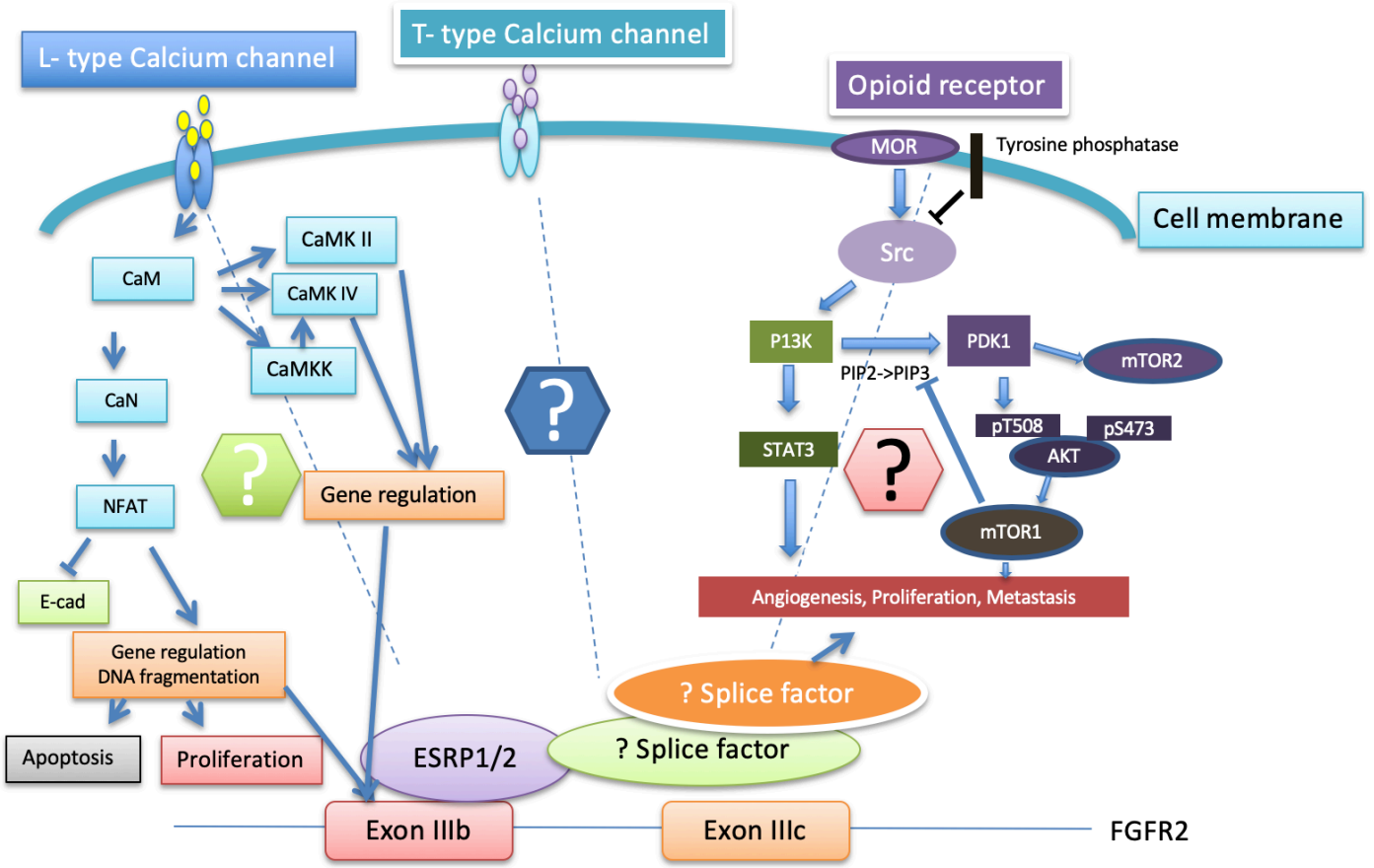


Figure 6-4 Potential LLSOs mechanism network

The LLSOs potential mechanism network based on splice factors and splicing signalling pathways. There are still some unknown key points involving the calcium channel or opioid receptor to splice the signalling pathway and involved in FGFR2 splicing.

6.6 Conclusion

In ESRP related xenograft experiments, ESRP1 knockdown in LNCaP, which is a more epithelial prostate cancer cell line, resulted in a significant increase in tumour growth in xenografts. ESRPs overexpression in PC3, which is a more mesenchymal prostate cancer cell line, resulted in a significant decrease in tumour growth. This suggests ESRPs may have tumour suppressor functions. It is worth seeing whether we could manipulate ESRPs functions.

In *in vitro* experiments, ESRPs decreased cell growth, affected EMT - induced E-cadherin, and decreased proliferation rate in PC3 cells, but did not affect migration. This revealed part of the way in which ESRPs could reduce tumour growth.

For the second aim – drug repurposing, I used *FGFR2* splicing-sensitive fluorescent reporter to screen chemicals from LOPAC library, found some possible modulators of EMT in *vitro*- LLSOs, which affect cell growth in different ways, decrease cell migration, and reduce proliferation rate in PC3 cells.

LLSO02 – Nemadipine-A at 10μM showed a significant decrease on tumour growth in the PC3 xenografts experiment.

As a further plan, I would like to explore the mechanisms of LLSOs: which splice factor is involved in *FGFR2* splicing and which signalling pathway is involved in EMT and tumour progression. Moreover, Nemadipine-A's effect on tumour metastasis could also be an interesting area to explore.

REFERENCES

Acloque, H., Adams, M. S., Fishwick, K., Bronner-Fraser, M., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions: the importance of changing cell state in development and disease. *Journal of Clinical Investigation, 119*(6), 1438-1449. doi:10.1172/jci38019

Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., . . . Venter, J. C. (2000). The genome sequence of Drosophila melanogaster. *Science, 287*(5461), 2185-2195. doi:10.1126/science.287.5461.2185

Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., & Clarke, M. F. (2003). Prospective identification of tumorigenic breast cancer cells. *Proceedings of the National Academy of Sciences of the United States of America, 100*(7), 3983-3988. doi:10.1073/pnas.0530291100

Al-Hasani, R., & Bruchas, M. R. (2011). Molecular Mechanisms of Opioid Receptor-dependent Signaling and Behavior. *Anesthesiology, 115*(6), 1363-1381. doi:10.1097/ALN.0b013e318238bba6

Alberti, C. (2016). Prostate cancer immunotherapy, particularly in combination with androgen deprivation or radiation treatment. Customized pharmacogenomic approaches to overcome immunotherapy cancer resistance. *Giornale Di Chirurgia, 37*(5), 225-235. doi:10.11138/gchir/2016.37.5.225

Alieva, A. K., Shadrina, M. I., Filatova, E. V., Karabanov, A. V., Illarioshkin, S. N., Limborska, S. A., & Slominsky, P. A. (2014). Involvement of Endocytosis and Alternative Splicing in the Formation of the Pathological Process in the Early Stages of Parkinson's Disease. *Biomed Research International*. doi:10.1155/2014/718732

Alt, F. W., Bothwell, A. L. M., Knapp, M., Siden, E., Mather, E., Koshland, M., & Baltimore, D. (1980). SYNTHESIS OF SECRETED AND MEMBRANE-BOUND IMMUNOGLOBULIN-MU HEAVY-CHAINS IS DIRECTED BY MESSENGER-RNAS THAT DIFFER AT THEIR 3' ENDS. *Cell, 20*(2), 293-301. doi:10.1016/0092-8674(80)90615-7

An, X., Sarmiento, C., Tan, T., & Zhu, H. (2017). Regulation of multidrug resistance by microRNAs in anti-cancer therapy. *Acta Pharmaceutica Sinica B, 7*(1), 38-51. doi:10.1016/j.apsb.2016.09.002

Anna, A., & Monika, G. (2018). Splicing mutations in human genetic disorders: examples, detection, and confirmation. *Journal of Applied Genetics, 59*(3), 253-268. doi:10.1007/s13353-018-0444-7

Arasada, R. R., Amann, J. M., Rahman, M. A., Huppert, S. S., & Carbone, D. P. (2014). EGFR Blockade Enriches for Lung Cancer Stem-like Cells through Notch3-Dependent Signaling. *Cancer Research, 74*(19), 5572-5584. doi:10.1158/0008-5472.can-13-3724

Armenia, J., Wankowicz, S. A. M., Liu, D., Gao, J. J., Kundra, R., Reznik, E., . . . Dre, P. S. C. I. P. C. (2018). The long tail of oncogenic drivers in prostate cancer. *Nature Genetics, 50*(5), 645-+. doi:10.1038/s41588-018-0078-z

Armstrong, A. J., Marengo, M. S., Oltean, S., Kemeny, G., Bitting, R. L., Turnbull, J. D., . . . Garcia-Blanco, M. A. (2011). Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. *Molecular Cancer Research, 9*(8), 997-1007. doi:10.1158/1541-7786.mcr-10-0490

Arslan, A. D., He, X. L., Wang, M. X., Rumschlag-Booms, E., Rong, L. J., & Beck, W. T. (2013). A High-Throughput Assay to Identify Small-Molecule Modulators of Alternative Pre-mRNA Splicing. *Journal of Biomolecular Screening, 18*(2), 180-190. doi:10.1177/1087057112459901

Auerbach, R., Lewis, R., Shinners, B., Kubai, L., & Akhtar, N. (2003). Angiogenesis assays: A critical overview. *Clinical Chemistry, 49*(1), 32-40. doi:10.1373/49.1.32

Autanski, D. B., Nagalingam, A., Bonner, M. Y., Arbiser, J. L., Saxena, N. K., & Sharma, D. (2014). Honokiol inhibits epithelial-mesenchymal transition in breast cancer cells by targeting signal transducer and activator of transcription 3/Zeb1/E-cadherin axis. *Molecular Oncology, 8*(3), 565-580. doi:10.1016/j.molonc.2014.01.004

Baade, P. D., Youlden, D. R., & Krnjacki, L. J. (2009). International epidemiology of prostate cancer: Geographical distribution and secular trends. *Molecular Nutrition & Food Research, 53*(2), 171-184. doi:10.1002/mnfr.200700511

Baraniak, A. P., Chen, J. R., & Garcia-Blanco, M. A. (2006). Fox-2 mediates epithelial cell-specific fibroblast growth factor receptor 2 exon choice. *Molecular and Cellular Biology, 26*(4), 1209-1222. doi:10.1128/mcb.26.4.1209-1222.2006

Barbieri, C. E., Baca, S. C., Lawrence, M. S., Demichelis, F., Blattner, M., Theurillat, J. P., . . . Garraway, L. A. (2012). Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. *Nature Genetics, 44*(6), 685-U107. doi:10.1038/ng.2279

Bates, D. O., Cui, T. G., Doughty, J. M., Winkler, M., Sugiono, M., Shields, J. D., . . . Harper, S. J. (2002). VEGF(165)b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma. *Cancer Research, 62*(14), 4123-4131.

Bates, D. O., & Curry, F. E. (1996). Vascular endothelial growth factor increases hydraulic conductivity of isolated perfused microvessels. *American Journal of Physiology-Heart and Circulatory Physiology, 271*(6), H2520-H2528.

Beadle, G. W., & Tatum, E. L. (1941). Genetic control of biochemical reactions in neurospora. *Proceedings of the National Academy of Sciences of the United States of America, 27*, 499-506. doi:10.1073/pnas.27.11.499

Beltran, H., Prandi, D., Mosquera, J. M., Benelli, M., Puca, L., Cyrta, J., . . . Demichelis, F. (2016). Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nature Medicine, 22*(3), 298-305. doi:10.1038/nm.4045

Berridge, M. J. (2012). Calcium signalling remodelling and disease. *Biochemical Society Transactions, 40*, 297-309. doi:10.1042/bst20110766

Berridge, M. V., & Tan, A. S. (1993). CHARACTERIZATION OF THE CELLULAR REDUCTION OF 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) - SUBCELLULAR-LOCALIZATION, SUBSTRATE DEPENDENCE, AND INVOLVEMENT OF MITOCHONDRIAL ELECTRON-TRANSPORT IN MTT REDUCTION. *Archives of Biochemistry and Biophysics, 303*(2), 474-482. doi:10.1006/abbi.1993.1311

Bevan, H. S., van den Akker, N. M. S., Qiu, Y., Polman, J. A. E., Foster, R. R., Yem, J., . . . Bates, D. O. (2008). The Alternatively Spliced Anti-Angiogenic Family of VEGF Isoforms VEGF(xxx)b in Human Kidney Development. *Nephron Physiology, 110*(4), 57-67. doi:10.1159/000177614

Biselli-Chicote, P. M., Oliveira, A., Pavarino, E. C., & Goloni-Bertollo, E. M. (2012). VEGF gene alternative splicing: pro- and anti-angiogenic isoforms in cancer. *Journal of Cancer Research and Clinical Oncology, 138*(3), 363-370. doi:10.1007/s00432-011-1073-2

Bosukonda, A., & Carlson, W. D. (2017). Harnessing the BMP signaling pathway to control the formation of cancer stem cells by effects on epithelial-to-mesenchymal transition. *Biochemical Society Transactions, 45*, 223-228. doi:10.1042/bst20160177

Boutet, A., De Frutos, C. A., Maxwell, P. H., Mayol, M. J., Romero, J., & Nieto, M. A. (2006). Snail activation disrupts tissue homeostasis and induces fibrosis in the adult kidney. *Embo Journal, 25*(23), 5603-5613. doi:10.1038/sj.emboj.7601421

Boyden, S. (1962). CHEMOTACTIC EFFECT OF MIXTURES OF ANTIBODY AND ANTIGEN ON POLYMORPHONUCLEAR LEUCOCYTES. *Journal of Experimental Medicine, 115*(3), 453-&. doi:10.1084/jem.115.3.453

Brabletz, T., Kalluri, R., Nieto, M. A., & Weinberg, R. A. (2018). EMT in cancer. *Nature Reviews Cancer, 18*(2), 128-+. doi:10.1038/nrc.2017.118

Braeutigam, C., Rago, L., Rolke, A., Waldmeier, L., Christofori, G., & Winter, J. (2014). The RNA-binding protein Rbfox2: an essential regulator of EMT-driven alternative splicing and a mediator of cellular invasion. *Oncogene, 33*(9), 1082-1092. doi:10.1038/onc.2013.50

Braunschweig, U., Barbosa-Morais, N. L., Pan, Q., Nachman, E. N., Alipanahi, B., Gonatopoulos-Pournatzis, T., . . . Blencowe, B. J. (2014). Widespread intron retention in mammals functionally tunes transcriptomes. *Genome Research, 24*(11), 1774-1786. doi:10.1101/gr.177790.114

Brower, V. (2016). Tasquinimod treatment for prostate cancer. *Lancet Oncology, 17*(8), E322-E322. doi:10.1016/s1470-2045(16)30272-8

Brzozowa, M., Michalski, M., Wyrobiec, G., Piecuch, A., Dittfeld, A., Harabin-Slowinska, M., . . . Wojnicz, R. (2015). The role of Snail1 transcription factor in colorectal cancer progression and metastasis. *Wspolczesna Onkologia-Contemporary Oncology, 19*(4), 265-270. doi:10.5114/wo.2014.42173

Bubendorf, L., Sauter, G., Moch, H., Jordan, P., Blochlinger, A., Gasser, T. C., & Mihatsch, M. J. (1996). Prognostic significance of Bcl-2 in clinically localized prostate cancer. *American Journal of Pathology, 148*(5), 1557-1565.

Buck, E., Eyzaguirre, A., Barr, S., Thompson, S., Sennello, R., Young, D., . . . Haley, J. D. (2007). Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Molecular Cancer Therapeutics, 6*(2), 532-541. doi:10.1158/1535-7163.mct-06-0462

BW, S., & CP, W. (2014). *World Cancer Report 2014*. Retrieved from

C, G., C, V., & G., B. (2008 Dec). Alternative splicing and tumor progression. *Current Genomics, 9*, 15. doi:10.2174/138920208786847971

Campos, C., Sotomayor, P., Jerez, D., Gonzalez, J., Schmidt, C. B., Schmidt, K., . . . Godoy, A. S. (2018). Exercise and prostate cancer: From basic science to clinical applications. *Prostate, 78*(9), 639-645. doi:10.1002/pros.23502

Caram, M. E. V., Estes, J. P., Griggs, J. J., Lin, P., & Mukherjee, B. (2018). Temporal and geographic variation in the systemic treatment of advanced prostate cancer. *Bmc Cancer, 18*, 10. doi:10.1186/s12885-018-4166-3

Carstens, R. P., Eaton, J. V., Krigman, H. R., Walther, P. J., & Garcia-Blanco, M. A. (1997). Alternative splicing of fibroblast growth factor receptor 2 (FGF-R2) in human prostate cancer. *Oncogene, 15*(25), 3059-3065. doi:10.1038/sj.onc.1201498

Carstens, R. P., Wagner, E. J., & Garcia-Blanco, M. A. (2000). An intronic splicing silencer causes skipping of the IIIb exon of fibroblast growth factor receptor 2 through involvement of polypyrimidine tract binding protein. *Molecular and Cellular Biology, 20*(19), 7388-7400. doi:10.1128/mcb.20.19.7388-7400.2000

Catz, S. D., & Johnson, J. L. (2003). BCL-2 in prostate cancer: A minireview. *Apoptosis, 8*(1), 29-37. doi:10.1023/a:1021692801278

Chaffer, C. L., & Weinberg, R. A. (2011). A Perspective on Cancer Cell Metastasis. *Science, 331*(6024), 1559-1564. doi:10.1126/science.1203543

Chaudhary, K. S., Abel, P. D., & Lalani, E. N. (1999). Role of the Bcl-2 gene family in prostate cancer progression and its implications for therapeutic intervention. *Environmental Health Perspectives, 107*, 49-57. doi:10.2307/3434471

Chen, I. T., Hsu, P. H., Hsu, W. C., Chen, N. J., & Tseng, P. H. (2015). Polyubiquitination of Transforming Growth Factor beta-activated Kinase 1 (TAK1) at Lysine 562 Residue Regulates TLR4-mediated JNK and p38 MAPK Activation. *Scientific Reports, 5*. doi:10.1038/srep12300

Chen, J., & Weiss, W. A. (2015). Alternative splicing in cancer: implications for biology and therapy. *Oncogene, 34*(1), 1-14. doi:10.1038/onc.2013.570

Chen, J. J., Guccini, I., Di Mitri, D., Brina, D., Revandkar, A., Sarti, M., . . . Alimonti, A. (2018). Compartmentalized activities of the pyruvate dehydrogenase complex sustain lipogenesis in prostate cancer. *Nature Genetics, 50*(2), 219-+. doi:10.1038/s41588-017-0026-3

Chen, L., Yao, Y., Sun, L. J., Zhou, J. J., Miao, M. M., Luo, S. J., . . . Tang, J. (2017). Snail Driving Alternative Splicing of CD44 by ESRP1 Enhances Invasion and Migration in Epithelial Ovarian Cancer. *Cellular Physiology and Biochemistry, 43*(6), 2489-2504. doi:10.1159/000484458

Chen, M., Zhang, J. W., Sampieri, K., Clohessy, J. G., Mendez, L., Gonzalez-Billalabeitia, E., . . . Pandolfi, P. P. (2018). An aberrant SREBP-dependent lipogenic program promotes metastatic prostate cancer. *Nature Genetics, 50*(2), 206-+. doi:10.1038/s41588-017-0027-2

Chen, T., You, Y. A., Jiang, H., & Wang, Z. Z. (2017). Epithelial-mesenchymal transition (EMT): A biological process in the development, stem cell differentiation, and tumorigenesis. *Journal of Cellular Physiology, 232*(12), 3261-3272. doi:10.1002/jcp.25797

Cheng, K. Y., & Hao, M. (2016). Metformin Inhibits TGF-beta 1-Induced Epithelial-to-Mesenchymal Transition via PKM2 Relative-mTOR/p70s6k Signaling Pathway in Cervical Carcinoma Cells. *International Journal of Molecular Sciences, 17*(12), 14. doi:10.3390/ijms17122000

Chow, L. T., Gelinas, R. E., Broker, T. R., & Roberts, R. J. (1977). An amazing sequence arrangement at the 5 ' ends of adenovirus 2 messenger RNA. *Cell, 12*(1), 1-8. doi:10.1016/0092-8674(77)90180-5

Chow, L. T., Roberts, J. M., Lewis, J. B., & Broker, T. R. (1977). Map of cytoplasmic RNA transcripts

from lytic adenovirus type-2, determined by electron-microscopy of RNA-DNA hybrids. *Cell, 11*(4), 819-836. doi:10.1016/0092-8674(77)90294-x

Consortium, C. e. S. (1998). Genome sequence of the nematode C-elegans: A platform for investigating biology. *Science, 282*(5396), 2012-2018. doi:10.1126/science.282.5396.2012

Cory, A. H., Owen, T. C., Barltrop, J. A., & Cory, J. G. (1991). USE OF AN AQUEOUS SOLUBLE TETRAZOLIUM FORMAZAN ASSAY FOR CELL-GROWTH ASSAYS IN CULTURE. *Cancer Communications, 3*(7), 207-212.

Cozar, J. M., Robles-Fernandez, I., Martinez-Gonzalez, L. J., Pascual-Geler, M., Rodriguez-Martinez, A., Serrano, M. J., . . . Alvarez-Cubero, M. J. (2018). Genetic markers a landscape in prostate cancer. *Mutation Research-Reviews in Mutation Research, 775*, 1-10. doi:10.1016/j.mrrev.2017.11.004

Crick, F. H., , : , v. 12, p. 138---63. (1958). On protein synthesis. In (Vol. 12, pp. 138-163): Symposia of the

Society for Experimental Biology.

Damato, R. J., Loughman, M. S., Flynn, E., & Folkman, J. (1994). THALIDOMIDE IS AN INHIBITOR OF ANGIOGENESIS. *Investigative Ophthalmology & Visual Science, 35*(4), 1996-1996.

Damianov, A., & Black, D. L. (2010). Autoregulation of Fox protein expression to produce dominant negative splicing factors. *Rna-a Publication of the Rna Society, 16*(2), 405-416. doi:10.1261/rna.1838210

Datta, A., Kim, H., McGee, L., Johnson, A. E., Talwar, S., Marugan, J., . . . Abdel-Mageed, A. B. (2018). High-throughput screening identified selective inhibitors of exosome biogenesis and secretion: A drug repurposing strategy for advanced cancer. *Scientific Reports, 8*. doi:10.1038/s41598-018-26411-7

David, C. J., Huang, Y. H., Chen, M., Su, J., Zou, Y. L., Bardeesy, N., . . . Massague, J. (2016). TGF-beta Tumor Suppression through a Lethal EMT. *Cell, 164*(5), 1015-1030. doi:10.1016/j.cell.2016.01.009

Davis, F. M., Azimi, I., Faville, R. A., Peters, A. A., Jalink, K., Putney, J. W., . . . Monteith, G. R. (2014). Induction of epithelial-mesenchymal transition (EMT) in breast cancer cells is calcium signal dependent. *Oncogene, 33*(18), 2307-2316. doi:10.1038/onc.2013.187

Davuluri, R. V., Suzuki, Y., Sugano, S., Plass, C., & Huang, T. H. M. (2008). The functional consequences of alternative promoter use in mammalian genomes. *Trends in Genetics, 24*(4), 167-177. doi:10.1016/j.tig.2008.01.008

De Craene, B., & Berx, G. (2013). Regulatory networks defining EMT during cancer initiation and progression. *Nature Reviews Cancer, 13*(2), 97-110. doi:10.1038/nrc3447

Del Gato-Konczak, F., Bourgeois, C. F., Le Guiner, C., Kister, L., Gesnel, M. C., Stevenin, J., & Breathnach, R. (2000). The RNA-binding protein TIA-1 is a novel mammalian splicing regulator acting through intron sequences adjacent to a 5 ' splice site. *Molecular and Cellular Biology, 20*(17), 6287-6299. doi:10.1128/mcb.20.17.6287-6299.2000

Del Gatto-Konczak, F., Olive, M., Gesnel, M. C., & Breathnach, R. (1999). hnRNP A1 recruited to an exon in vivo can function as an exon splicing silencer. *Molecular and Cellular Biology, 19*(1), 251-260.

Delgatto, F., & Breathnach, R. (1995). EXON AND INTRON SEQUENCES, RESPECTIVELY, REPRESS AND ACTIVATE SPLICING OF A FIBROBLAST GROWTH-FACTOR RECEPTOR-2 ALTERNATIVE EXON. *Molecular and Cellular Biology, 15*(9), 4825-4834.

DelGatto, F., Gesnel, M. C., & Breathnach, R. (1996). The exon sequence TAGG can inhibit splicing. *Nucleic Acids Research, 24*(11), 2017-2021. doi:10.1093/nar/24.11.2017

Deng, X. Y., Apple, S., Zhao, H., Song, J., Lee, M., Luo, W., . . . Chang, H. R. (2017). CD24 Expression and differential resistance to chemotherapy in triple-negative

breast cancer. *Oncotarget, 8*(24), 38294-38308. doi:10.18632/oncotarget.16203

Di, C. X., Syafrizayanti, Zhang, Q. J., Chen, Y. H., Wang, Y. P., Zhang, X. T., . . . Hoheisel, J. D. (2019). Function, clinical application, and strategies of Pre-mRNA splicing in cancer. *Cell Death and Differentiation, 26*(7), 1181-1194. doi:10.1038/s41418-018-0231-3

Diaz-Lopez, A., Moreno-Bueno, G., & Cano, A. (2014). Role of microRNA in epithelial to mesenchymal transition and metastasis and clinical perspectives. *Cancer Management and Research, 6*, 205-216. doi:10.2147/cmar.s38156

Dillard, P. R., Lin, M. F., & Khan, S. A. (2008). Androgen-independent prostate cancer cells acquire the complete steroidogenic potential of synthesizing testosterone from cholesterol. *Molecular and Cellular Endocrinology, 295*(1-2), 115-120. doi:10.1016/j.mce.2008.08.013

Dittmar, K. A., Jiang, P., Park, J. W., Amirikian, K., Wan, J., Shen, S., . . . Carstens, R. P. (2012). Genome-Wide Determination of a Broad ESRP-Regulated Posttranscriptional Network by High-Throughput Sequencing. *Molecular and Cellular Biology, 32*(8), 1468-1482. doi:10.1128/mcb.06536-11

Djulbegovic, M., Beyth, R. J., Neuberger, M. M., Stoffs, T. L., Vieweg, J., Djulbegovic, B., & Dahm, P. (2010). Screening for prostate cancer: systematic review and meta-analysis of randomised controlled trials. *Bmj-British Medical Journal, 341*, 9. doi:10.1136/bmj.c4543

Du, B. W., & Shim, J. S. (2016). Targeting Epithelial-Mesenchymal Transition (EMT) to Overcome Drug Resistance in Cancer. *Molecules, 21*(7). doi:10.3390/molecules21070965

Eswarakumar, V. P., Lax, I., & Schlessinger, J. (2005). Cellular signaling by fibroblast growth factor receptors. *Cytokine & Growth Factor Reviews, 16*(2), 139-149. doi:10.1016/j.cytogfr.2005.01.001

Ewing, B., & Green, P. (2000). Analysis of expressed sequence tags indicates 35,000 human genes. *Nature Genetics, 25*(2), 232-234.

Ezkurdia, I., Juan, D., Rodriguez, J. M., Frankish, A., Diekhans, M., Harrow, J., . . . Tress, M. L. (2014). Multiple evidence strands suggest that there may be as few as 19 000 human protein-coding genes. *Human Molecular Genetics, 23*(22), 5866-5878. doi:10.1093/hmg/ddu309

Fagoonee, S., Picco, G., Orso, F., Arrigoni, A., Longo, D. L., Forni, M., . . . Altruda, F. (2017). The RNA-binding protein ESRP1 promotes human colorectal cancer progression. *Oncotarget, 8*(6), 10007-10024. doi:10.18632/oncotarget.14318

Faustino, N. A., & Cooper, T. A. (2003). Pre-mRNA splicing and human disease. *Genes & Development, 17*(4), 419-437. doi:10.1101/gad.1048803

Feng, H. J., Qin, Z. Y., & Zhang, X. G. (2013). Opportunities and methods for studying alternative splicing in cancer with RNA-Seq. *Cancer Letters, 340*(2), 179-191. doi:10.1016/j.canlet.2012.11.010

Ferrara, N., Houck, K. A., Jakeman, L. B., Winer, J., & Leung, D. W. (1991). THE VASCULAR ENDOTHELIAL GROWTH-FACTOR FAMILY OF POLYPEPTIDES. *Journal of Cellular Biochemistry, 47*(3), 211-218. doi:10.1002/jcb.240470305

Fleshner, K., Carlsson, S. V., & Roobol, M. J. (2017). The effect of the USPSTF PSA screening recommendation on prostate cancer incidence patterns in the USA. *Nature Reviews Urology, 14*(1), 26-37. doi:10.1038/nrurol.2016.251

Gamazon, E. R., & Stranger, B. E. (2014). Genomics of alternative splicing: evolution, development and pathophysiology. *Human Genetics, 133*(6), 679-687. doi:10.1007/s00439-013-1411-3

Gao, H., Korn, J. M., Ferretti, S., Monahan, J. E., Wang, Y. Z., Singh, M., . . . Sellers, W. R. (2015). High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nature Medicine, 21*(11), 1318-1325. doi:10.1038/nm.3954

Gao, X. L., Jin, Q. J., Jiang, C., Li, Y., Li, C. H., Liu, H. Q., . . . Xu, J. R. (2016). FgPrp4 Kinase Is Important for Spliceosome B-Complex Activation and Splicing Efficiency in Fusarium graminearum. *Plos Genetics, 12*(4), 23. doi:10.1371/journal.pgen.1005973

Gatta, G., Mallone, S., van der Zwan, J. M., Trama, A., Siesling, S., Capocaccia, R., & Grp, E. W. (2013). Cancer prevalence estimates in Europe at the beginning of 2000. *Annals of Oncology, 24*(6), 1660-1666. doi:10.1093/annonc/mdt030

Goncalves, V., Matos, P., & Jordan, P. (2009). Antagonistic SR proteins regulate alternative splicing of tumor-related Rac1b downstream of the PI3-kinase and Wnt pathways. *Human Molecular Genetics, 18*(19), 3696-3707. doi:10.1093/hmg/ddp317

Gonzalez, D. M., & Medici, D. (2014). Signaling mechanisms of the epithelial-mesenchymal transition. *Science Signaling, 7*(344). doi:10.1126/scisignal.2005189

Grasso, C. S., Wu, Y. M., Robinson, D. R., Cao, X. H., Dhanasekaran, S. M., Khan, A. P., . . . Tomlins, S. A. (2012). The mutational landscape of lethal castration-resistant prostate cancer. *Nature, 487*(7406), 239-243. doi:10.1038/nature11125

Graveley, B. R. (2001). Alternative splicing: increasing diversity in the proteomic world. *Trends in Genetics, 17*(2), 100-107. doi:10.1016/s0168-9525(00)02176-4

Grygielko, E. T., Martin, W. M., Tweed, C., Thornton, P., Harling, J., Brooks, D. P., & Laping, N. J. (2005). Inhibition of gene markers of fibrosis with a novel inhibitor of transforming growth factor-beta type I receptor kinase in puromycin-induced nephritis. *Journal of Pharmacology and Experimental Therapeutics, 313*(3), 943-951. doi:10.1124/jpet.104.082099

Guo, W. J., Keckesova, Z., Donaher, J. L., Shibue, T., Tischler, V., Reinhardt, F., . . . Weinberg, R. A. (2012). Slug and Sox9 Cooperatively Determine the Mammary Stem Cell State. *Cell, 148*(5), 1015-1028. doi:10.1016/j.cell.2012.02.008

Gupta, N., Al Ustwani, O., Shen, L., & Pili, R. (2014). Mechanism of action and clinical activity of tasquinimod in castrate-resistant prostate cancer. *Oncotargets and Therapy, 7*, 223-234. doi:10.2147/ott.s53524

Guy, J. B., Espenel, S., Vallard, A., Battiston-Montagne, P., Wozny, A. S., Ardail, D., . . . Magne, N. (2017). Evaluation of the Cell Invasion and Migration Process: A Comparison of the Video Microscope-based Scratch Wound Assay and the Boyden Chamber Assay. *Jove-Journal of Visualized Experiments*(129), 7. doi:10.3791/56337

Han, D., Wu, G., Chang, C., Zhu, F., Xiao, Y., Li, Q. H., . . . Zhang, L. L. (2015). Disulfiram inhibits TGF-beta-induced epithelial-mesenchymal transition and stem-like features in breast cancer via ERK/NF-kappa B/Snail pathway. *Oncotarget, 6*(38), 40907-40919. doi:10.18632/oncotarget.5723

Harari, S., Elia, D., Torre, O., Bulgheroni, E., Provasi, E., & Moss, J. (2016). Sirolimus Therapy for Patients With Lymphangioleiomyomatosis Leads to Loss of Chylous Ascites and Circulating LAM Cells. *Chest, 150*(2), E29-E32. doi:10.1016/j.chest.2016.02.654

Harper, S. J., & Bates, D. O. (2008). VEGF-A splicing: the key to anti-angiogenic therapeutics? *Nature Reviews Cancer, 8*(11), 880-887. doi:10.1038/nrc2505

Hay, E. D. (1995). An overview of epithelio-mesenchymal transformation. *Acta Anatomica, 154*(1), 8-20.

Hayakawa, A., Saitoh, M., & Miyazawa, K. (2017). Dual Roles for Epithelial Splicing Regulatory Proteins 1 (ESRP1) and 2 (ESRP2) in Cancer Progression. *Advances in experimental medicine and biology, 925*, 33-40. doi:10.1007/5584_2016_50

Hayes, G. M., Carrigan, P. E., Beck, A. M., & Miller, L. J. (2006). Targeting the RNA splicing machinery as a novel treatment strategy for pancreatic carcinoma. *Cancer Research, 66*(7), 3819-3827. doi:10.1158/0008-5472.can-05-4065

Heinlein, C. A., & Chang, C. S. (2004). Androgen receptor in prostate cancer. *Endocrine Reviews, 25*(2), 276-308. doi:10.1210/er.2002-0032

Heldin, C. H., & Moustakas, A. (2012). Role of Smads in TGF beta signaling. *Cell and Tissue Research, 347*(1), 21-36. doi:10.1007/s00441-011-1190-x

Helsten, T., Schwaederle, M., & Kurzrock, R. (2015). Fibroblast growth factor receptor signaling in hereditary and neoplastic disease: biologic and clinical implications. *Cancer and Metastasis Reviews, 34*(3), 479-496. doi:10.1007/s10555-015-9579-8

Hema, S., Thambiraj, S., & Shankaran, D. R. (2018). Nanoformulations for Targeted Drug Delivery to Prostate Cancer: An Overview. *Journal of Nanoscience and Nanotechnology, 18*(8), 5171-5191. doi:10.1166/jnn.2018.15420

Hollier, B. G., Tinnirello, A. A., Werden, S. J., Evans, K. W., Taube, J. H., Sarkar, T. R., . . . Mani, S. A. (2013). FOXC2 Expression Links Epithelial-Mesenchymal Transition and Stem Cell Properties in Breast Cancer. *Cancer Research, 73*(6), 1981-1992. doi:10.1158/0008-5472.can-12-2962

Horiguchi, K., Sakamoto, K., Koinuma, D., Semba, K., Inoue, A., Inoue, S., . . . Saitoh, M. (2012). TGF-beta drives epithelial-mesenchymal transition through delta EF1-mediated downregulation of ESRP. *Oncogene, 31*(26), 3190-3201. doi:10.1038/onc.2011.493

Hovhannisyan, R. H., & Carstens, R. P. (2007). Heterogeneous ribonucleoprotein M is a splicing regulatory protein that can enhance or silence splicing of alternatively spliced Exons. *Journal of Biological Chemistry, 282*(50), 36265-36274. doi:10.1074/jbc.M704188200

Hovhannisyan, R. H., Warzecha, C. C., & Carstens, R. P. (2006). Characterization of sequences and mechanisms through which ISE/ISS-3 regulates FGFR2 splicing. *Nucleic Acids Research, 34*(1), 373-385. doi:10.1093/nar/gkj407

Hsu, H. Y., Lin, T. Y., Hwang, P. A., Tseng, L. M., Chen, R. H., Tsao, S. M., & Hsu, J. (2013). Fucoidan induces changes in the epithelial to mesenchymal transition and decreases metastasis by enhancing ubiquitin-dependent TGF beta receptor degradation in breast cancer. *Carcinogenesis, 34*(4), 874-884. doi:10.1093/carcin/bgs396

Hu, J., Li, G., Zhang, P. Y., Zhuang, X. Q., & Hu, G. H. (2017). A CD44v(+) subpopulation of breast cancer stem-like cells with enhanced lung metastasis capacity. *Cell Death & Disease, 8*, 9. doi:10.1038/cddis.2017.72

Huang, L. H., & Fu, L. W. (2015). Mechanisms of resistance to EGFR tyrosine kinase inhibitors. *Acta Pharmaceutica Sinica B, 5*(5), 390-401. doi:10.1016/j.apsb.2015.07.001

Huntsman, D. G., & Caldas, C. (1998). Assignment of the E-cadherin gene (CDH1) to chromosome 16q22.1 by radiation hybrid mapping. *Cytogenetics and Cell Genetics, 83*(1-2), 82-83. doi:10.1159/000015134

Huttenhain, R., Soste, M., Selevsek, N., Rost, H., Sethi, A., Carapito, C., . . . Aebersold, R. (2012). Reproducible Quantification of Cancer-Associated Proteins in Body Fluids Using Targeted Proteomics. *Science Translational Medicine, 4*(142). doi:10.1126/scitranslmed.3003989

Institute, N. C. (2018). Cancer Stat Facts: Prostate Cancer.

Ishii, H., Saitoh, M., Sakamoto, K., Kondo, T., Katoh, R., Tanaka, S., . . . Miyazawa, K. (2014). Epithelial Splicing Regulatory Proteins 1 (ESRP1) and 2 (ESRP2) Suppress Cancer Cell Motility via Different Mechanisms. *The Journal of biological chemistry, 289*(40), 27386-27399. doi:10.1074/jbc.M114.589432

Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H., & Neilson, E. G. (2002). Evidence that fibroblasts derive from epithelium during tissue fibrosis. *Journal of Clinical Investigation, 110*(3), 341-350. doi:10.1172/jci200215518

Jakobsen, K. R., Demuth, C., Sorensen, B. S., & Nielsen, A. L. (2016). The role of epithelial to mesenchymal transition in resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. *Translational Lung Cancer Research, 5*(2), 172-182. doi:10.21037/tlcr.2016.04.07

Jeong, H. M., Han, J., Lee, S. H., Park, H. J., Lee, H. J., Choi, J. S., . . . Kwon, M. J. (2017). ESRP1 is overexpressed in ovarian cancer and promotes switching from mesenchymal to epithelial phenotype in ovarian cancer cells (vol 6, e389, 2017). *Oncogenesis, 6*. doi:10.1038/oncsis.2017.89

Jha, G. G., Gupta, S., Tagawa, S. T., Koopmeiners, J. S., Vivek, S., Dudek, A. Z., . . . Miller, J. S. (2017). A phase II randomized, double-blind study of sipuleucel-T followed by IDO pathway inhibitor, indoximod, or placebo in the treatment of patients with metastatic castration resistant prostate cancer (mCRPC). *Journal of Clinical Oncology, 35*, 5. doi:10.1200/JCO.2017.35.15_suppl.3066

Jin, C. Y., Moon, D. O., Lee, J. D., Heo, M. S., Choi, Y. H., Lee, C. M., . . . Kim, G. Y. (2007). Sulforaphane sensitizes tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis through downregulation of ERK and Akt in lung adenocarcinoma A549 cells. *Carcinogenesis, 28*(5), 1058-1066. doi:10.1093/carcin/bgl251

Jolly, M. K., Ware, K. E., Gilja, S., Somarelli, J. A., & Levine, H. (2017). EMT and MET: necessary or permissive for metastasis? *Molecular Oncology, 11*(7), 755-769. doi:10.1002/1878-0261.12083

Jorand, R., Biswas, S., Wakefield, D. L., Tobin, S. J., Golfetto, O., Hilton, K., . . . Jovanovic-Talisman, T. (2016). Molecular signatures of mu opioid receptor and somatostatin receptor 2 in pancreatic cancer. *Molecular Biology of the Cell, 27*(22), 3659-3672. doi:10.1091/mbc.E16-06-0427

Joshi, G., Singh, P. K., Negi, A., Rana, A., Singh, S., & Kumar, R. (2015). Growth factors mediated cell signalling in prostate cancer progression: Implications in discovery of anti-prostate cancer agents. *Chemico-Biological Interactions, 240*, 120-133. doi:10.1016/j.cbi.2015.08.009

Joy, M. E., Vollmer, L. L., Hulkower, K., Stern, A. M., Peterson, C. K., Boltz, R. C., . . . Vogt, A. (2014). A High-Content, Multiplexed Screen in Human Breast Cancer Cells Identifies Profilin-1 Inducers with Anti-Migratory Activities. *Plos One, 9*(2), 12. doi:10.1371/journal.pone.0088350

Justus, C. R., Leffler, N., Ruiz-Echevarria, M., & Yang, L. V. (2014). In vitro Cell Migration and Invasion Assays. *Jove-Journal of Visualized Experiments*(88), 8. doi:10.3791/51046

Kalluri, R. (2009). EMT: When epithelial cells decide to become mesenchymal-like cells. *Journal of Clinical Investigation, 119*(6), 1417-1419. doi:10.1172/jci39675

Kalluri, R., & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *Journal of Clinical Investigation, 112*(12), 1776-1784. doi:10.1172/jci200320530

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *Journal of Clinical Investigation, 119*(6), 1420-1428. doi:10.1172/jci39104

Kang, Y. B., & Pantel, K. (2013). Tumor Cell Dissemination: Emerging Biological Insights from Animal Models and Cancer Patients. *Cancer Cell, 23*(5), 573-581. doi:10.1016/j.ccr.2013.04.017

Kantoff, P. W., Higano, C. S., Shore, N. D., Berger, E. R., Small, E. J., Penson, D. F., . . . Investigators, I. S. (2010). Sipuleucel-T Immunotherapy for Castration-Resistant Prostate Cancer. *New England Journal of Medicine, 363*(5), 411-422. doi:10.1056/NEJMoa1001294

Katoh, M. (2009). FGFR2 Abnormalities Underlie a Spectrum of Bone, Skin, and Cancer Pathologies. *Journal of Investigative Dermatology, 129*(8), 1861-1867. doi:10.1038/jid.2009.97

Katoh, M. (2016). FGFR inhibitors: Effects on cancer cells, tumor microenvironment and whole-body homeostasis (Review). *International Journal of Molecular Medicine, 38*(1), 3-15. doi:10.3892/ijmm.2016.2620

Katoh, Y., & Katoh, M. (2009). FGFR2-related pathogenesis and FGFR2-targeted therapeutics. *International Journal of Molecular Medicine, 23*(3), 307-311. doi:10.3892/ijmm_00000132

Kendall, R. T., & Feghali-Bostwick, C. A. (2014). Fibroblasts in fibrosis: novel roles and mediators. *Frontiers in Pharmacology, 5*. doi:10.3389/fphar.2014.00123

Kim, E., Goren, A., & Ast, G. (2008). Insights into the connection between cancer and alternative splicing. *Trends in Genetics, 24*(1), 7-10. doi:10.1016/j.tig.2007.10.001

Kim, K. K., Kim, Y. C., Adelstein, R. S., & Kawamoto, S. (2011). Fox-3 and PSF interact to activate neural cell-specific alternative splicing. *Nucleic Acids Research, 39*(8), 3064-3078. doi:10.1093/nar/gkq1221

Kim, Y.-J., & Kim, H.-S. (2012). Alternative Splicing and Its Impact as a Cancer Diagnostic Marker. *Genomics & Informatics, 10*(2), 74-80. doi:10.5808/gi.2012.10.2.74

Kolawole, A. O., Hixon, B. P., Dameron, L. S., Chrisman, I. M., & Smirnov, V. V. (2015). Catalytic activity of human indoleamine 2,3-dioxygenase (hIDO1) at low oxygen. *Archives of Biochemistry and Biophysics, 570*, 47-57. doi:10.1016/j.abb.2015.02.014

Kole, R., Krainer, A. R., & Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nature Reviews Drug Discovery, 11*(2), 125-140. doi:10.1038/nrd3625

Konarska, M. M., Grabowski, P. J., Padgett, R. A., & Sharp, P. A. (1985). CHARACTERIZATION OF THE BRANCH SITE IN LARIAT RNAS PRODUCED BY SPLICING OF MESSENGER-RNA PRECURSORS. *Nature, 313*(6003), 552-557. doi:10.1038/313552a0

Koo, V., El Mekabaty, A., Hamilton, P., Maxwell, P., Sharaf, O., Diamond, J., . . . Williamson, K. (2010). Novel in vitro assays for the characterization of EMT in tumourigenesis. *Cellular Oncology, 32*(1-2), 67-76. doi:10.3233/clo-2009-0501

Korpal, M., Lee, E. S., Hu, G. H., & Kang, Y. B. (2008). The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *Journal of Biological Chemistry, 283*(22), 14910-14914. doi:10.1074/jbc.C800074200

Kramer, N., Walzl, A., Unger, C., Rosner, M., Krupitza, G., Hengstschlager, M., & Dolznig, H. (2013). In vitro cell migration and invasion assays. *Mutation Research-Reviews in Mutation Research, 752*(1), 10-24. doi:10.1016/j.mrrev.2012.08.001

Krawczak, M., Reiss, J., & Cooper, D. N. (1992). THE MUTATIONAL SPECTRUM OF SINGLE BASE-PAIR SUBSTITUTIONS IN MESSENGER-RNA SPLICE JUNCTIONS OF HUMAN GENES - CAUSES AND CONSEQUENCES. *Human Genetics, 90*(1-2), 41-54.

Ku, D. D., Zaleski, J. K., Liu, S. Y., & Brock, T. A. (1993). VASCULAR ENDOTHELIAL GROWTH-FACTOR INDUCES EDRF-DEPENDENT RELAXATION IN CORONARY-ARTERIES. *American Journal of Physiology, 265*(2), H586-H592.

Kulka, J., Szekely, B., Lukacs, L. V., Kiss, O., Tokes, A. M., Vincze, E., . . . Szasz, A. M. (2016). Comparison of Predictive Immunohistochemical Marker Expression of Primary Breast Cancer and Paired Distant Metastasis using Surgical Material: A Practice-Based Study. *Journal of Histochemistry & Cytochemistry, 64*(4), 256-267. doi:10.1369/0022155416639013

Kuzumaki, N., Suzuki, A., Narita, M., Hosoya, T., Nagasawa, A., Imai, S., . . . Suzuki, T. (2012). Effect of kappa-opioid receptor agonist on the growth of non-small cell lung cancer (NSCLC) cells. *British Journal of Cancer, 106*(6), 1148-1152. doi:10.1038/bjc.2011.574

Kypta, R. M., & Waxman, J. (2012). Wnt/beta-catenin signalling in prostate cancer. *Nature Reviews Urology, 9*(8), 418-428. doi:10.1038/nrurol.2012.116

Lamouille, S., Xu, J., & Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. *Nature Reviews Molecular Cell Biology, 15*(3), 178-196. doi:10.1038/nrm3758

Lareau, L. F., Green, R. E., Bhatnagar, R. S., & Brenner, S. E. (2004). The evolving roles of alternative splicing. *Current Opinion in Structural Biology, 14*(3), 273-282. doi:10.1016/j.sbi.2004.05.002

Lauffenburger, D. A., & Horwitz, A. F. (1996). Cell migration: A physically integrated molecular process. *Cell, 84*(3), 359-369. doi:10.1016/s0092-8674(00)81280-5

Lee, A. F., Chen, M. C., Chen, C. J., Yang, C. J., Huang, M. S., & Liu, Y. P. (2017). Reverse epithelial-mesenchymal transition contributes to the regain of drug sensitivity in tyrosine kinase inhibitor-resistant non-small cell lung cancer cells. *Plos One, 12*(7). doi:10.1371/journal.pone.0180383

Lee, J. M., Dedhar, S., Kalluri, R., & Thompson, E. W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *Journal of Cell Biology, 172*(7), 973-981. doi:10.1083/jcb.200601018

Lee, S. W., Kwak, H. S., Kang, M. H., Park, Y. Y., & Jeong, G. S. (2018). Fibroblast-associated tumour microenvironment induces vascular structure-networked tumouroid. *Scientific Reports, 8*. doi:10.1038/s41598-018-20886-0

Lennnon, F. E., Mirzapoiazova, T., Mambetsariev, B., Salgia, R., Moss, J., & Singleton, P. A. (2012). Overexpression of the mu opioid receptor in human non-small cell

lung cancer promotes Akt and mTOR activation, tumor growth and metastasis. *Cancer Research, 72*, 1. doi:10.1158/1538-7445.am2012-2232

Lennon, F. E., Mirzapoiazova, T., Mambetsariev, B., Poroyko, V. A., Salgia, R., Moss, J., & Singleton, P. A. (2014). The Mu Opioid Receptor Promotes Opioid and Growth Factor-Induced Proliferation, Migration and Epithelial Mesenchymal Transition (EMT) in Human Lung Cancer. *Plos One, 9*(3), 13. doi:10.1371/journal.pone.0091577

Lertwittayapon, T., Tencomnao, T., & Santiyanont, R. (2012). Inhibitory effect of alternatively spliced RAGEv1 on the expression of NF-kappa B and TNF-alpha in hepatocellular carcinoma cells. *Genetics and Molecular Research, 11*(2), 1712-1720. doi:10.4238/2012.June.29.3

Lewis, B. P., Green, R. E., & Brenner, S. E. (2003). Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proceedings of the National Academy of Sciences of the United States of America, 100*(1), 189-192. doi:10.1073/pnas.0136770100

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., . . . Parsons, R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science, 275*(5308), 1943-1947. doi:10.1126/science.275.5308.1943

Li, R., Dong, T. T., Hu, C., Lu, J. J., Dai, J., & Liu, P. S. (2017). Salinomycin repressed the epithelial-mesenchymal transition of epithelial ovarian cancer cells via downregulating Wnt/beta-catenin pathway. *Oncotargets and Therapy, 10*, 1317-1325. doi:10.2147/ott.s126463

Licatalosi, D. D., & Darnell, R. B. (2006). Splicing regulation in neurologic disease. *Neuron, 52*(1), 93-101. doi:10.1016/j.neuron.2006.09.017

Lima, A. R., Araujo, A. M., Pinto, J., Jeronimo, C., Henrique, R., Bastos, M. D., . . . de Pinho, P. G. (2018). Discrimination between the human prostate normal and cancer cell exometabolome by GC-MS. *Scientific Reports, 8*, 12. doi:10.1038/s41598-018-23847-9

Lin, Y. T., Fukuchi, J., Hiipakka, R. A., Kokontis, J. M., & Xiang, J. L. (2007). Up-regulation of Bcl-2 is required for the progression of prostate cancer cells from an androgen-dependent to an androgen-independent growth stage. *Cell Research, 17*(6), 531-536. doi:10.1038/cr.2007.12

Ling, L., & Oltean, S. (2017). Modulators of alternative splicing as novel therapeutics in cancer. *International Journal of Molecular Medicine, 40*, S23-S23.

Liu, Z. Y., Wang, Y. H., Wang, S. L., Zhang, J., Zhang, F., & Niu, Y. (2012). Nek2C functions as a tumor promoter in human breast tumorigenesis. *International Journal of Molecular Medicine, 30*(4), 775-782. doi:10.3892/ijmm.2012.1069

Lorch, A. (2017). Systemic treatment of metastatic prostate cancer. *Onkologe, 23*(3), 237-250. doi:10.1007/s00761-016-0132-3

Lotz-Jenne, C., Luthi, U., Ackerknecht, S., Lehembre, F., Fink, T., Stritt, M., . . . Meyer-Schaller, N. (2016). A high-content EMT screen identifies multiple receptor

tyrosine kinase inhibitors with activity on TGF beta receptor. *Oncotarget, 7*(18), 25983-26002. doi:10.18632/oncotarget.8418

Loughlin, K. R. (2014). Calcium channel blockers and prostate cancer. *Urologic Oncology-Seminars and Original Investigations, 32*(5), 537-538. doi:10.1016/j.urolonc.2013.08.001

Lu, C. M., Huguley, S., Cui, C., Cabaniss, L. B., Waite, P. D., Sarver, D. M., . . . MacDougall, M. (2015). Effects of FGFR Signaling on Cell Proliferation and Differentiation of Apert Dental Cells. *Cells Tissues Organs, 201*(1), 26-37. doi:10.1159/000441349

Lu, Z. X., Huang, Q., Park, J. W., Shen, S. H., Lin, L., Tokheim, C. J., . . . Xing, Y. (2015). Transcriptome-wide Landscape of Pre-mRNA Alternative Splicing Associated with Metastatic Colonization. *Molecular Cancer Research, 13*(2), 305-318. doi:10.1158/1541-7786.mcr-14-0366

Lunde, B. M., Moore, C., & Varani, G. (2007). RNA-binding proteins: modular design for efficient function. *Nature Reviews Molecular Cell Biology, 8*(6), 479-490. doi:10.1038/nrm2178

Lundholt, B. K., Scudder, K. M., & Pagliaro, L. (2003). A simple technique for reducing edge effect in cell-based assays. *Journal of Biomolecular Screening, 8*(5), 566-570. doi:10.1177/1087057103256465

Macarron, R., Banks, M. N., Bojanic, D., Burns, D. J., Cirovic, D. A., Garyantes, T., . . . Sittampalam, G. S. (2011). Impact of high-throughput screening in biomedical research. *Nature Reviews Drug Discovery, 10*(3), 188-195. doi:10.1038/nrd3368

Mahdi, S. H. A., Cheng, H. Y., Li, J. F., & Feng, R. Q. (2015). The effect of TGF-beta-induced epithelial mesenchymal transition on the expression of intracellular calcium-handling proteins in T47D and MCF-7 human breast cancer cells. *Archives of Biochemistry and Biophysics, 583*, 18-26. doi:10.1016/j.abb.2015.07.008

Mahmood, T., & Yang, P.-C. (2012). Western blot: technique, theory, and trouble shooting. *North American journal of medical sciences, 4*(9), 429-434. doi:10.4103/1947-2714.100998

Maia, M. C., & Hansen, A. R. (2017). A comprehensive review of immunotherapies in prostate cancer. *Critical Reviews in Oncology Hematology, 113*, 292-303. doi:10.1016/j.critrevonc.2017.02.026

Malek, R., Wang, H., Kekoa, T. D., & Tran, P. T. (2017). Therapeutic Targeting of Epithelial Plasticity Programs: Focus on the Epithelial-Mesenchymal Transition. *Cells Tissues Organs, 203*(2), 114-127. doi:10.1159/000447238

Malo, N., Hanley, J. A., Cerquozzi, S., Pelletier, J., & Nadon, R. (2006). Statistical practice in high-throughput screening data analysis. *Nature Biotechnology, 24*(2), 167-175. doi:10.1038/nbt1186

Mancini, M., & Toker, A. (2009). NFAT proteins: emerging roles in cancer progression. *Nature Reviews Cancer, 9*(11), 810-820. doi:10.1038/nrc2735

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., . . . Weinberg, R. A. (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell, 133*(4), 704-715. doi:10.1016/j.cell.2008.03.027

Maquat, L. E., & Carmichael, G. G. (2001). Quality control of mRNA function. *Cell, 104*(2), 173-176. doi:10.1016/s0092-8674(01)00202-1

Marcucci, F., Stassi, G., & De Maria, R. (2016). Epithelial-mesenchymal transition: a new target in anticancer drug discovery. *Nature Reviews Drug Discovery, 15*(5), 311-325. doi:10.1038/nrd.2015.13

Matsuda, Y., Ueda, J., & Ishiwata, T. (2012). Fibroblast growth factor receptor 2: expression, roles, and potential as a novel molecular target for colorectal cancer. *Pathology research international, 2012*, 574768. doi:10.1155/2012/574768

Mauger, D. M., Lin, C., & Garcia-Blanco, M. A. (2008). HnRNP H and hnRNP F complex with Fox2 to silence fibroblast growth factor receptor 2 Exon IIIc. *Molecular and Cellular Biology, 28*(17), 5403-5419. doi:10.1128/mcb.00739-08

Maxam, A. M., & Gilbert, W. (1977). NEW METHOD FOR SEQUENCING DNA. *Proceedings of the National Academy of Sciences of the United States of America, 74*(2). doi:10.1073/pnas.74.2.560

McManus, C. J., & Graveley, B. R. (2011). RNA structure and the mechanisms of alternative splicing. *Current Opinion in Genetics & Development, 21*(4), 373-379. doi:10.1016/j.gde.2011.04.001

Michelini, E., Cevenini, L., Mezzanotte, L., Coppa, A., & Roda, A. (2010). Cell-based assays: fuelling drug discovery. *Analytical and Bioanalytical Chemistry, 398*(1), 227-238. doi:10.1007/s00216-010-3933-z

Mitchell, C. A., Rutland, C. S., Walker, M., Nasir, M., Foss, A. J. E., Stewart, C., . . . Drexler, H. C. A. (2006). Unique vascular phenotypes following over-expression of individual VEGFA isoforms from the developing lens. *Angiogenesis, 9*(4), 209-224. doi:10.1007/s10456-006-9056-7

Miura, K., Fujibuchi, W., & Sasaki, I. (2011). Alternative pre-mRNA splicing in digestive tract malignancy. *Cancer Science, 102*(2), 309-316. doi:10.1111/j.1349-7006.2010.01797.x

Mladinich, M., Ruan, D., & Chan, C. H. (2016). Tackling Cancer Stem Cells via Inhibition of EMT Transcription Factors. *Stem Cells International*, 10. doi:10.1155/2016/5285892

Modrek, B., & Lee, C. (2002). A genomic view of alternative splicing. *Nature Genetics, 30*(1), 13-19. doi:10.1038/ng0102-13

Moghadam, M. J. F., Taheri, S., & Peiravian, F. (2018). A Systematic Review of Clinical Practice Guidelines for Castration-Resistant Prostate Cancer. *Iranian Journal of Pharmaceutical Research, 17*, 17-37.

Montemurro, F., Valabrega, G., & Aglietta, M. (2007). Lapatinib: a dual inhibitor of EGFR and HER2 tyrosine kinase activity. *Expert Opinion on Biological Therapy, 7*(2), 257-268. doi:10.1517/14712598.7.2.257

Morton, C. L., & Houghton, P. J. (2007). Establishment of human tumor xenografts in immunodeficient mice. *Nature Protocols, 2*(2), 247-250. doi:10.1038/nprot.2007.25

Morton, J. J., Bird, G., Refaeli, Y., & Jimeno, A. (2016). Humanized Mouse Xenograft Models: Narrowing the Tumor-Microenvironment Gap. *Cancer Research, 76*(21), 6153-6158. doi:10.1158/0008-5472.can-16-1260

Mosmann, T. (1983). RAPID COLORIMETRIC ASSAY FOR CELLULAR GROWTH AND SURVIVAL - APPLICATION TO PROLIFERATION AND CYTO-TOXICITY ASSAYS. *Journal of Immunological Methods, 65*(1-2), 55-63. doi:10.1016/0022-1759(83)90303-4

Moyer, V. A., & Force, U. S. P. S. T. (2012). Screening for Prostate Cancer: US Preventive Services Task Force Recommendation Statement. *Annals of Internal Medicine, 157*(2), 120-+. doi:10.7326/0003-4819-157-2-201207170-00459

Muh, S. J., Hovhannisyan, R. H., & Carstens, R. P. (2002). A non-sequence-specific double-stranded RNA structural element regulates splicing of two mutually exclusive exons of fibroblast growth factor receptor 2 (FGFR2). *Journal of Biological Chemistry, 277*(51), 50143-50154. doi:10.1074/jbc.M207409200

Nabhan, C., Patel, A., Villines, D., Tolzien, K., Kelby, S. K., & Lestingi, T. M. (2014). Lenalidomide Monotherapy in Chemotherapy-Naive, Castration-Resistant Prostate Cancer Patients: Final Results of a Phase II Study. *Clinical Genitourinary Cancer, 12*(1), 27-32. doi:10.1016/j.clgc.2013.09.001

Namba, T. (2015). Zidovudine, an anti-viral drug, resensitizes gemcitabine-resistant pancreatic cancer cells to gemcitabine by inhibition of the Akt-GSK3beta-Snail pathway. *European Journal of Cancer, 51*, S62-S62. doi:10.1016/s0959-8049(16)30186-1

Naryshkin, N. A., Weetall, M., Dakka, A., Narasimhan, J., Zhao, X., Feng, Z. H., . . . Metzger, F. (2014). SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy. *Science, 345*(6197), 688-693. doi:10.1126/science.1250127

Newman, E. A., Muh, S. J., Hovhannisyan, R. H., Warzecha, C. C., Jones, R. B., McKeehan, W. L., & Carstens, R. P. (2006). Identification of RNA-binding proteins that regulate FGFR2 splicing through the use of sensitive and specific dual color fluorescence minigene assays. *Rna-a Publication of the Rna Society, 12*(6), 1129-1141. doi:10.1261/rna.34906

Nguyen, D. P., Li, J. Y., Yadav, S. S., & Tewari, A. K. (2014). Recent insights into NF-kappa B signalling pathways and the link between inflammation and prostate cancer. *Bju International, 114*(2), 168-176. doi:10.1111/bju.12488

Nygren, P., Fryknas, M., Anagel, B., & Larsson, R. (2013). Repositioning of the anthelmintic drug mebendazole for the treatment for colon cancer. *Journal of*

Cancer Research and Clinical Oncology, 139(12), 2133-2140. doi:10.1007/s00432-013-1539-5

Okegawa, T., Ninomiya, N., Masuda, K., Nakamura, Y., Tambo, M., & Nutahara, K. (2018). AR-V7 in circulating tumor cells cluster as a predictive biomarker of abiraterone acetate and enzalutamide treatment in castration-resistant prostate cancer patients. *Prostate, 78*(8), 576-582. doi:10.1002/pros.23501

Oltean, S., & Bates, D. O. (2014). Hallmarks of alternative splicing in cancer. *Oncogene, 33*(46), 5311-5318. doi:10.1038/onc.2013.533

Oltean, S., Febbo, P. G., & Garcia-Blanco, M. A. (2008). Dunning rat prostate adenocarcinomas and alternative splicing reporters: powerful tools to study epithelial plasticity in prostate tumors in vivo. *Clinical & Experimental Metastasis, 25*(6), 611-619. doi:10.1007/s10585-008-9186-y

Oltean, S., Sorg, B. S., Albrecht, T., Bonano, V. I., Brazas, R. M., Dewhirst, M. W., & Garcia-Blanco, M. A. (2006). Alternative inclusion of fibroblast growth factor receptor 2 exon IIIc in Dunning prostate tumors reveals unexpected epithelial mesenchymal plasticity. *Proceedings of the National Academy of Sciences of the United States of America, 103*(38), 14116-14121. doi:10.1073/pnas.0603090103

Orengo, J. P., Bundman, D., & Cooper, T. A. (2006). A bichromatic fluorescent reporter for cell-based screens of alternative splicing. *Nucleic Acids Research, 34*(22). doi:10.1093/nar/gkl967

Ornitz, D. M., Xu, J. S., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., . . . Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *Journal of Biological Chemistry, 271*(25), 15292-15297. doi:10.1074/jbc.271.25.15292

Orrurtreger, A., Bedford, M. T., Burakova, T., Arman, E., Zimmer, Y., Yayon, A., . . . Lonai, P. (1993). DEVELOPMENTAL LOCALIZATION OF THE SPLICING ALTERNATIVES OF FIBROBLAST GROWTH-FACTOR RECEPTOR-2 (FGFR2). *Developmental Biology, 158*(2), 475-486. doi:10.1006/dbio.1993.1205

Pajares, M. J., Ezponda, T., Catena, R., Calvo, A., Pio, R., & Montuenga, L. M. (2007). Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncology, 8*(4), 349-357. doi:10.1016/s1470-2045(07)70104-3

Papaemmanuil, E., Cazzola, M., Boultwood, J., Malcovati, L., Vyas, P., Bowen, D., . . . Int Canc Genome, C. (2011). Somatic SF3B1 Mutation in Myelodysplasia with Ring Sideroblasts. *New England Journal of Medicine, 365*(15), 1384-1395. doi:10.1056/NEJMoa1103283

Paradis, C., Cloutier, P., Shkreta, L., Toutant, J., Klarskov, K., & Chabot, B. (2007). HnRNP I/PTB can antagonize the splicing repressor activity of SRp30c. *Rna-a Publication of the Rna Society, 13*(8), 1287-1300. doi:10.1261/rna.403607

Park, S. H., Park, S. J., Kim, J. O., Shin, J. H., Kim, E. S., Jo, Y. K., . . . Cho, D. H. (2013). Down-Regulation of Survivin by Nemadipine-A Sensitizes Cancer Cells to TRAIL-

Induced Apoptosis. *Biomolecules & Therapeutics, 21*(1), 29-34. doi:10.4062/biomolther.2012.088

Pennisi, E. (2000). Human genome project - And the gene number is...? *Science, 288*(5469), 1146-1147. doi:10.1126/science.288.5469.1146

Pereira, D. A., & Williams, J. A. (2007). Origin and evolution of high throughput screening. *British Journal of Pharmacology, 152*(1), 53-61. doi:10.1038/sj.bjp.0707373

Pettitt, S. J., Krastev, D. B., Brandsma, I., Drean, A., Song, F. F., Aleksandrov, R., . . . Lord, C. J. (2018). Genome-wide and high-density CRISPR-Cas9 screens identify point mutations in PARP1 causing PARP inhibitor resistance. *Nature Communications, 9*. doi:10.1038/s41467-018-03917-2

Platz, E. A., Yegnasubramanian, S., Liu, J. O., Chong, C. R., Shim, J. S., Kenfield, S. A., . . . Nelson, W. G. (2011). A Novel Two-Stage, Transdisciplinary Study Identifies Digoxin as a Possible Drug for Prostate Cancer Treatment. *Cancer Discovery, 1*(1), 68-77. doi:10.1158/2159-8274.cd-10-0020

Polireddy, K., Dong, R., McDonald, P. R., Wang, T., Luke, B., Chen, P., . . . Chen, Q. (2016). Targeting Epithelial-Mesenchymal Transition for Identification of Inhibitors for Pancreatic Cancer Cell Invasion and Tumor Spheres Formation. *Plos One, 11*(10). doi:10.1371/journal.pone.0164811

Pratilas, C. A., Xing, F., & Solit, D. B. (2012). Targeting oncogenic BRAF in human cancer. *Current topics in microbiology and immunology, 355*, 83-98. doi:10.1007/82_2011_162

Pritchard, C. C., Mateo, J., Walsh, M. F., De Sarkar, N., Abida, W., Beltran, H., . . . Nelson, P. S. (2016). Inherited DNA-Repair Gene Mutations in Men with Metastatic Prostate Cancer. *New England Journal of Medicine, 375*(5), 443-453. doi:10.1056/NEJMoa1603144

PS, R., & JP, R. (2002 May). *Overview of functional cell assays*: Curr Protoc Cytom.

Quinn, D. I., Shore, N. D., Egawa, S., Gerritsen, W. R., & Fizazi, K. (2015). Immunotherapy for castration-resistant prostate cancer: Progress and new paradigms. *Urologic Oncology-Seminars and Original Investigations, 33*(5), 245-260. doi:10.1016/j.urolonc.2014.10.009

Rajan, P., Elliott, D. J., Robson, C. N., & Leung, H. Y. (2009). Alternative splicing and biological heterogeneity in prostate cancer. *Nature Reviews Urology, 6*(8), 454-460. doi:10.1038/nrurol.2009.125

Ranieri, D., Rosato, B., Nanni, M., Magenta, A., Belleudi, F., & Torrisi, M. R. (2016). Expression of the FGFR2 mesenchymal splicing variant in epithelial cells drives epithelial-mesenchymal transition. *Oncotarget, 7*(5), 5440-5460. doi:10.18632/oncotarget.6706

Reka, A. K., Kuick, R., Kurapati, H., Standiford, T. J., Omenn, G. S., & Keshamouni, V. G. (2011). Identifying Inhibitors of Epithelial-Mesenchymal Transition by Connectivity Map-Based Systems Approach. *Journal of Thoracic Oncology, 6*(11), 1784-1792. doi:10.1097/JTO.0b013e31822adfb0

Richmond, A., & Su, Y. J. (2008). Mouse xenograft models vs GEM models for human cancer therapeutics. *Disease Models & Mechanisms, 1*(2-3), 78-82. doi:10.1242/dmm.000976

Robson, E. J. D., Khaled, W. T., Abell, K., & Watson, C. J. (2006). Epithelial-to-mesenchymal transition confers resistance to apoptosis in three murine mammary epithelial cell lines. *Differentiation, 74*(5), 254-264. doi:10.1111/j.1432-0436.2006.00075.x

Salton, M., & Misteli, T. (2016). Small Molecule Modulators of Pre-mRNA Splicing in Cancer Therapy. *Trends in Molecular Medicine, 22*(1), 28-37. doi:10.1016/j.molmed.2015.11.005

Santamaria, P. G., Moreno-Bueno, G., Portillo, F., & Cano, A. (2017). EMT: Present and future in clinical oncology. *Molecular Oncology, 11*(7), 718-738. doi:10.1002/1878-0261.12091

Sato, M., Muragaki, Y., Saika, S., Roberts, A. B., & Ooshima, A. (2003). Targeted disruption of TGF-beta 1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *Journal of Clinical Investigation, 112*(10), 1486-1494. doi:10.1172/jci200319270

Scanlon, C. S., Van Tubergen, E. A., Inglehart, R. C., & D'Silva, N. J. (2013). Biomarkers of Epithelial-Mesenchymal Transition in Squamous Cell Carcinoma. *Journal of Dental Research, 92*(2), 114-121. doi:10.1177/0022034512467352

Schreiner, D., Simicevic, J., Ahrne, E., Schmidt, A., & Scheiffele, P. (2015). Quantitative isoform-profiling of highly diversified recognition molecules. *Elife, 4*. doi:10.7554/eLife.07794

Seo, J., Shin, J. Y., Leijten, J., Jeon, O., Camci-Unal, G., Dikina, A. D., . . . Khademhosseini, A. (2018). High-throughput approaches for screening and analysis of cell behaviors. *Biomaterials, 153*, 85-101. doi:10.1016/j.biomaterials.2017.06.022

Shapiro, I. M., Cheng, A. W., Flytzanis, N. C., Balsamo, M., Condeelis, J. S., Oktay, M. H., . . . Gertler, F. B. (2011). An EMT-Driven Alternative Splicing Program Occurs in Human Breast Cancer and Modulates Cellular Phenotype. *Plos Genetics, 7*(8). doi:10.1371/journal.pgen.1002218

Shapovalov, G., Skryma, R., & Prevarskaya, N. (2013). Calcium Channels and Prostate Cancer. *Recent Patents on Anti-Cancer Drug Discovery, 8*(1), 18-26.

Shen, L., Sundstedt, A., Ciesielski, M., Miles, K. M., Celander, M., Adelaiye, R., . . . Pili, R. (2015). Tasquinimod Modulates Suppressive Myeloid Cells and Enhances Cancer Immunotherapies in Murine Models. *Cancer Immunology Research, 3*(2), 136-148. doi:10.1158/2326-6066.cir-14-0036

Shi, Y. G. (2017). The Spliceosome: A Protein-Directed Metalloribozyme. *Journal of Molecular Biology, 429*(17), 2640-2653. doi:10.1016/j.jmb.2017.07.010

Shiga, K., Hara, M., Nagasaki, T., Sato, T., Takahashi, H., & Takeyama, H. (2015). Cancer-Associated Fibroblasts: Their Characteristics and Their Roles in Tumor Growth. *Cancers, 7*(4), 2443-2458. doi:10.3390/cancers7040902

Shin, J. A., Hong, O. K., Lee, H. J., Jeon, S. Y., Kim, J. W., Lee, S. H., . . . Yoon, K. H. (2011). Transforming Growth Factor-beta Induces Epithelial to Mesenchymal Transition and Suppresses the Proliferation and Transdifferentiation of Cultured Human Pancreatic Duct Cells. *Journal of Cellular Biochemistry, 112*(1), 179-188. doi:10.1002/jcb.22929

Shoag, J., & Barbieri, C. E. (2016). Clinical variability and molecular heterogeneity in prostate cancer. *Asian Journal of Andrology, 18*(4), 543-548. doi:10.4103/1008-682x.178852

Siebzehnrubl, F. A., Silver, D. J., Tugertimur, B., Deleyrolle, L. P., Siebzehnrubl, D., Sarkisian, M. R., . . . Steindler, D. A. (2013). The ZEB1 pathway links glioblastoma initiation, invasion and chemoresistance. *Embo Molecular Medicine, 5*(8), 1196-1212. doi:10.1002/emmm.201302827

Siegfried, Z., & Karni, R. (2018). The role of alternative splicing in cancer drug resistance. *Current Opinion in Genetics & Development, 48*, 16-21. doi:10.1016/j.gde.2017.10.001

Silipo, M., Gautrey, H., & Tyson-Capper, A. (2015). Deregulation of splicing factors and breast cancer development. *Journal of Molecular Cell Biology, 7*(5), 388-401. doi:10.1093/jmcb/mjv027

Silvestri, I., Cattarino, S., Giantulli, S., Nazzari, C., Collalti, G., & Sciarra, A. (2016). A Perspective of Immunotherapy for Prostate Cancer. *Cancers, 8*(7). doi:10.3390/cancers8070064

Singh, A., & Settleman, J. (2010). EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. *Oncogene, 29*(34), 4741-4751. doi:10.1038/onc.2010.215

Singh, R. K., & Cooper, T. A. (2012). Pre-mRNA splicing in disease and therapeutics. *Trends in Molecular Medicine, 18*(8), 472-482. doi:10.1016/j.molmed.2012.06.006

Singhal, S., Mehta, J., Desikan, R., Ayers, D., Roberson, P., Eddlemon, P., . . . Crowley, J. (1999). Antitumor activity of thalidomide in refractory multiple myeloma. *New England Journal of Medicine, 341*(21), 1565-1571. doi:10.1056/nejm199911183412102

Singleton, P. A., Moss, J., Karp, D. D., Atkins, J. T., & Janku, F. (2015). The Mu Opioid Receptor: A New Target for Cancer Therapy? *Cancer, 121*(16), 2681-2688. doi:10.1002/cncr.29460

Slaugenhaupt, S. A., Blumenfeld, A., Gill, S. P., Leyne, M., Mull, J., Cuajungco, M. P., . . . Gusella, J. F. (2001). Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. *American Journal of Human Genetics, 68*(3), 598-605. doi:10.1086/318810

Spillantini, M. G. (2003). Tau gene mutations in frontotemporal dementia and parkinsonism linked to chromosome 17. *International Seminar on Nuclear War and Planetary Emergencies - 27th Session*, 647-659. doi:10.1142/9789812705150_0071

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K. A., Lin, H., Ligon, A. H., . . . Tavtigian, S. V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nature Genetics, 15*(4), 356-362. doi:10.1038/ng0497-356

Sterne-Weiler, T., & Sanford, J. R. (2014). Exon identity crisis: disease-causing mutations that disrupt the splicing code. *Genome Biology, 15*(1). doi:10.1186/gb4150

Stewart BW, W. C. (2014). *World Cancer Report 2014*. Retrieved from

Stoilov, P., Lin, C. H., Damoiseaux, R., Nikolic, J., & Black, D. L. (2008). A high-throughout screening strategy identifies cardiotonic steroids as alternative splicing modulators. *Proceedings of the National Academy of Sciences of the United States of America, 105*(32), 11218-11223. doi:10.1073/pnas.0801661105

Stratton, J., & Godwin, M. (2011). The effect of supplemental vitamins and minerals on the development of prostate cancer: a systematic review and meta-analysis. *Family Practice, 28*(3), 243-252. doi:10.1093/fampra/cmq115

Strauss, R., Hamerlik, P., Lieber, A., & Bartek, J. (2012). Regulation of Stem Cell Plasticity: Mechanisms and Relevance to Tissue Biology and Cancer. *Molecular Therapy, 20*(5), 887-897. doi:10.1038/mt.2012.2

Sumanasekera, C., Kelemen, O., Beullens, M., Aubol, B. E., Adams, J. A., Sunkara, M., . . . Stamm, S. (2012). C6 pyridinium ceramide influences alternative pre-mRNA splicing by inhibiting protein phosphatase-1. *Nucleic Acids Research, 40*(9), 4025-4039. doi:10.1093/nar/gkr1289

Sun, S. Y., Zhang, Z., Sinha, R., Karni, R., & Krainer, A. R. (2010). SF2/ASF autoregulation involves multiple layers of post-transcriptional and translational control. *Nature Structural & Molecular Biology, 17*(3), 306-U370. doi:10.1038/nsmb.1750

Sune-Pou, M., Prieto-Sanchez, S., Boyero-Corral, S., Moreno-Castro, C., El Yousfi, Y., Sune-Negre, J. M., . . . Sune, C. (2017). Targeting Splicing in the Treatment of Human Disease. *Genes, 8*(3). doi:10.3390/genes8030087

Tang, J., Ahmad, A., & Sarkar, F. H. (2012). The Role of MicroRNAs in Breast Cancer Migration, Invasion and Metastasis. *International Journal of Molecular Sciences, 13*(10), 13414-13437. doi:10.3390/ijms131013414

Tania, M., Khan, M. A., & Fu, J. J. (2014). Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. *Tumor Biology, 35*(8), 7335-7342. doi:10.1007/s13277-014-2163-y

Tarkkonen, K. M., Nilsson, E. M., Kahkonen, T. E., Dey, J. H., Heikkila, J. E., Tuomela, J. M., . . . Harkonen, P. L. (2012). Differential Roles of Fibroblast Growth Factor Receptors (FGFR) 1, 2 and 3 in the Regulation of S115 Breast Cancer Cell Growth. *Plos One, 7*(11). doi:10.1371/journal.pone.0049970

Taylor, B. S., Schultz, N., Hieronymus, H., Gopalan, A., Xiao, Y. H., Carver, B. S., . . . Gerald, W. L. (2010). Integrative Genomic Profiling of Human Prostate Cancer. *Cancer Cell, 18*(1), 11-22. doi:10.1016/j.ccr.2010.05.026

Thiery, J. P., Acloque, H., Huang, R. Y. J., & Nieto, M. A. (2009). Epithelial-Mesenchymal Transitions in Development and Disease. *Cell, 139*(5), 871-890. doi:10.1016/j.cell.2009.11.007

Thiery, J. P., & Sleeman, J. P. (2006). Complex networks orchestrate epithelial-mesenchymal transitions. *Nature Reviews Molecular Cell Biology, 7*(2), 131-142. doi:10.1038/nrm1835

Tian, D., Zhu, M., Chen, W. S., Li, J. S., Wu, R. L., & Wang, X. (2006). Role of glycogen synthase kinase 3 in squamous differentiation induced by cigarette smoke in porcine tracheobronchial epithelial cells. *Food and Chemical Toxicology, 44*(9), 1590-1596. doi:10.1016/j.fct.2006.03.013

Tian, L. J., Duncan, R. R., Hammond, M. S. L., Coghill, L. S., Wen, H., Rusinova, R., . . . Shipston, M. J. (2001). Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *Journal of Biological Chemistry, 276*(11), 7717-7720. doi:10.1074/jbc.C000741200

Tran, D. D., Corsa, C. A. S., Biswas, H., Aft, R. L., & Longmore, G. D. (2011). Temporal and Spatial Cooperation of Snail1 and Twist1 during Epithelial-Mesenchymal Transition Predicts for Human Breast Cancer Recurrence. *Molecular Cancer Research, 9*(12), 1644-1657. doi:10.1158/1541-7786.mcr-11-0371

Ueda, J., Matsuda, Y., Yamahatsu, K., Uchida, E., Naito, Z., Korc, M., & Ishiwata, T. (2014). Epithelial splicing regulatory protein 1 is a favorable prognostic factor in pancreatic cancer that attenuates pancreatic metastases. *Oncogene, 33*(36), 4485-4495. doi:10.1038/onc.2013.392

Venables, J. P. (2004). Aberrant and alternative splicing in cancer. *Cancer Research, 64*(21), 7647-7654. doi:10.1158/0008-5472.can-04-1910

Venables, J. P. (2006). Unbalanced alternative splicing and its significance in cancer. *Bioessays, 28*(4), 378-386. doi:10.1002/bies.20390

Villaronga, M. A., Bevan, C. L., & Belandia, B. (2008). Notch Signaling: A Potential Therapeutic Target in Prostate Cancer. *Current Cancer Drug Targets, 8*(7), 566-580. doi:10.2174/156800908786241096

Voon, D. C., Huang, R. Y., Jackson, R. A., & Thiery, J. P. (2017). The EMT spectrum and therapeutic opportunities. *Molecular Oncology, 11*(7), 878-891. doi:10.1002/1878-0261.12082

Wagner, E. J., Baraniak, A. P., Sessions, O. M., Mauger, D., Moskowitz, E., & Garcia-Blanco, M. A. (2005). Characterization of the intronic splicing silencers flanking FGFR2 exon IIIb. *Journal of Biological Chemistry, 280*(14), 14017-14027. doi:10.1074/jbc.M414492200

Wagner, E. J., & Garcia-Blanco, M. A. (2001). Polypyrimidine tract binding protein antagonizes exon definition. *Molecular and Cellular Biology, 21*(10), 3281-3288. doi:10.1128/mcb.21.10.3281-3288.2001

Wagner, E. J., & Garcia-Blanco, M. A. (2002). RNAi-mediated PTB depletion leads to enhanced exon definition. *Molecular Cell, 10*(4), 943-949. doi:10.1016/s1097-2765(02)00645-7

Wahl, G. M., & Spike, B. T. (2017). Cell state plasticity, stem cells, EMT, and the generation of intra-tumoral heterogeneity. *Npj Breast Cancer, 3*. doi:10.1038/s41523-017-0012-z

Wahl, M. C., & Luhrmann, R. (2015). SnapShot: Spliceosome Dynamics III. *Cell, 162*(3), 2. doi:10.1016/j.cell.2015.07.033

Wang, E. T., Sandberg, R., Luo, S. J., Khrebtukova, I., Zhang, L., Mayr, C., . . . Burge, C. B. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature, 456*(7221), 470-476. doi:10.1038/nature07509

Wang, G. C., Zhao, D., Spring, D. J., & DePinho, R. A. (2018). Genetics and biology of prostate cancer. *Genes & Development, 32*(17-18), 1105-1140. doi:10.1101/gad.315739.118

Wang, S. L., Li, W. D., Liu, N., Zhang, F., Liu, H., Liu, F., . . . Niu, Y. (2012). Nek2A contributes to tumorigenic growth and possibly functions as potential therapeutic target for human breast cancer. *Journal of Cellular Biochemistry, 113*(6), 1904-1914. doi:10.1002/jcb.24059

Wang, X. L., Shi, W. P., Shi, H. C., Lu, S. C., Wang, K., Sun, C., . . . Shu, Y. S. (2016). TRIM11 overexpression promotes proliferation, migration and invasion of lung cancer cells. *Journal of Experimental & Clinical Cancer Research, 35*. doi:10.1186/s13046-016-0379-y

Wang, Y., Chen, D., Qian, H. L., Tsai, Y. H. S., Shao, S. J., Liu, Q. T., . . . Wang, Z. F. (2014). The Splicing Factor RBM4 Controls Apoptosis, Proliferation, and Migration to Suppress Tumor Progression. *Cancer Cell, 26*(3), 374-389. doi:10.1016/j.ccr.2014.07.010

Wang, Y. F., Shi, J., Chai, K. Q., Ying, X. H., & Zhou, B. H. P. (2013). The Role of Snail in EMT and Tumorigenesis. *Current Cancer Drug Targets, 13*(9), 963-972. doi:10.2174/15680096113136660102

Warzecha, C. C., & Carstens, R. P. (2012). Complex changes in alternative pre-mRNA splicing play a central role in the epithelial-to-mesenchymal transition (EMT). *Seminars in Cancer Biology, 22*(5-6), 417-427. doi:10.1016/j.semcancer.2012.04.003

Warzecha, C. C., Jiang, P., Amirikian, K., Dittmar, K. A., Lu, H. Z., Shen, S. H., . . . Carstens, R. P. (2010). An ESRP-regulated splicing programme is abrogated during the epithelial-mesenchymal transition. *Embo Journal, 29*(19), 3286-3300. doi:10.1038/emboj.2010.195

Warzecha, C. C., Sato, T. K., Nabet, B., Hogenesch, J. B., & Carstens, R. P. (2009). ESRP1 and ESRP2 Are Epithelial Cell-Type-Specific Regulators of FGFR2 Splicing. *Molecular Cell, 33*(5), 591-601. doi:10.1016/j.molcel.2009.01.025

Warzecha, C. C., Shen, S., Xing, Y., & Carstens, R. P. (2009). The epithelial splicing factors ESRP1 and ESRP2 positively and negatively regulate diverse types of alternative splicing events. *Rna Biology, 6*(5), 546-562.

Welch, H. G., & Black, W. C. (2010). Overdiagnosis in Cancer. *Journal of the National Cancer Institute, 102*(9), 605-613. doi:10.1093/jnci/djq099

Whang, Y. E., Armstrong, A. J., Rathmell, W. K., Godley, P. A., Kim, W. Y., Pruthi, R. S., . . . George, D. J. (2013). A phase II study of lapatinib, a dual EGFR and HER-2 tyrosine kinase inhibitor, in patients with castration-resistant prostate cancer. *Urologic Oncology-Seminars and Original Investigations, 31*(1), 82-86. doi:10.1016/j.urolonc.2010.09.018

Wilkinson, G. F., & Pritchard, K. (2015). In Vitro Screening for Drug Repositioning. *Journal of Biomolecular Screening, 20*(2), 167-179. doi:10.1177/1087057114563024

Wong, Y. N. S., Ferraldeschi, R., Attard, G., & de Bono, J. (2014). Evolution of androgen receptor targeted therapy for advanced prostate cancer. *Nature Reviews Clinical Oncology, 11*(6), 365-376. doi:10.1038/nrclinonc.2014.72

Xia, S. J., Ji, R., & Zhan, W. W. (2017). Long noncoding RNA papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3) inhibits proliferation and invasion of glioma cells by suppressing the Wnt/beta-catenin signaling pathway. *Bmc Neurology, 17*. doi:10.1186/s12883-017-0813-6

Xu, J., Lamouille, S., & Derynck, R. (2009). TGF-beta-induced epithelial to mesenchymal transition. *Cell Research, 19*(2), 156-172. doi:10.1038/cr.2009.5

Xu, Q., Deng, F., Qin, Y., Zhao, Z., Wu, Z., Xing, Z., . . . Wang, Q. J. (2016). Long non-coding RNA regulation of epithelial-mesenchymal transition in cancer metastasis. *Cell Death & Disease, 7*. doi:10.1038/cddis.2016.149

Yang, A. L., Zhao, Y. A., Wang, Y., Zha, X. J., Zhao, Y. F., Tu, P. F., & Hu, Z. D. (2018). Huaier suppresses proliferative and metastatic potential of prostate cancer PC3 cells via downregulation of Lamin B1 and induction of autophagy. *Oncology Reports, 39*(6), 3055-3063. doi:10.3892/or.2018.6358

Yang, J., He, D., Peng, Y., Zhong, H. Z., Deng, Y. H., Yu, Z. H., . . . Xu, Z. M. (2017). Matrine suppresses the migration and invasion of NSCLC cells by inhibiting PAX2-induced epithelial-mesenchymal transition. *Oncotargets and Therapy, 10*, 5209-5217. doi:10.2147/ott.s149609

Yang, S. S., Zhang, Y. Y., Meng, F. L., Liu, Y. D., Xia, B. R., Xiao, M., . . . Lou, G. (2013). Overexpression of multiple myeloma SET domain (MMSET) is associated with advanced tumor aggressiveness and poor prognosis in serous ovarian carcinoma. *Biomarkers, 18*(3), 257-263. doi:10.3109/1354750x.2013.773082

Yang, Y. Q., Park, J. W., Bebee, T. W., Warzecha, C. C., Guo, Y., Shang, X. Q., . . . Carstens, R. P. (2016). Determination of a Comprehensive Alternative Splicing Regulatory Network and Combinatorial Regulation by Key Factors during the Epithelial-to-Mesenchymal Transition. *Molecular and Cellular Biology, 36*(11), 1704-1719. doi:10.1128/mcb.00019-16

Yap, T. A., Smith, A. D., Ferraldeschi, R., Al-Lazikani, B., Workman, P., & de Bono, J. S. (2016). Drug discovery in advanced prostate cancer: translating biology into therapy. *Nature Reviews Drug Discovery, 15*(10), 699-718. doi:10.1038/nrd.2016.120

Yasumoto, H., Matsubara, A., Mutaguchi, K., Usui, T., & McKeehan, W. L. (2004). Restoration of fibroblast growth factor receptor2 suppresses growth and tumorigenicity of malignant human prostate carcinoma PC-3 cells. *Prostate, 61*(3), 236-242. doi:10.1002/pros.20093

Yi, E. Y., Park, S. Y., Jung, S. Y., Jang, W. J., & Kim, Y. J. (2015). Mitochondrial dysfunction induces EMT through the TGF-beta/Smad/Snail signaling pathway in Hep3B hepatocellular carcinoma cells. *International Journal of Oncology, 47*(5), 1845-1853. doi:10.3892/ijo.2015.3154

Zago, G., Biondini, M., Camonis, J., & Parrini, M. C. (2017). A family affair: A Ral-exocyst-centered network links Ras, Rac, Rho signaling to control cell migration. *Small GTPases*, 1-8. doi:10.1080/21541248.2017.1310649

Zavadil, J., Haley, J., Kalluri, R., Muthuswamy, S. K., & Thompson, E. (2008). Epithelial-Mesenchymal Transition. *Cancer Research, 68*(23), 9574-9577. doi:10.1158/0008.5472.can-08-2316

Zeisberg, M., & Duffield, J. S. (2010). Resolved: EMT Produces Fibroblasts in the Kidney. *Journal of the American Society of Nephrology, 21*(8), 1247-1253. doi:10.1681/asn.2010060616

Zhang, X., Wei, C. Y., Li, J., Liu, J. L., & Qu, J. Q. (2017). MicroRNA-361-5p inhibits epithelial-to-mesenchymal transition of glioma cells through targeting Twist1. *Oncology Reports, 37*(3), 1849-1856. doi:10.3892/or.2017.5406

Zhang, X. Q., Ibrahimi, O. A., Olsen, S. K., Umemori, H., Mohammadi, M., & Ornitz, D. M. (2006). Receptor specificity of the fibroblast growth factor family - The complete mammalian FGF family. *Journal of Biological Chemistry, 281*(23), 15694-15700. doi:10.1074/jbc.M601252200

Zhao, Q., Caballero, O. L., Davis, I. D., Jonasch, E., Tamboli, P., Yung, W. K. A., . . . Network, T. R. (2013). Tumor-Specific Isoform Switch of the Fibroblast Growth Factor Receptor 2 Underlies the Mesenchymal and Malignant Phenotypes of Clear Cell Renal Cell Carcinomas. *Clinical Cancer Research, 19*(9), 2460-2472. doi:10.1158/1078-0432.ccr-12-3708

Zhou, Y.-j., Shi, J., Tian, G., Zhou, H.-x., & Gao, G.-q. (2016). Effect of neferine on proliferation and invasion of human hepatocellular carcinoma cell line HepG2 and Bel-7402. *Chinese Pharmacological Bulletin, 32*(11), 1539-1542. doi:10.3969/j.issn.1001-1978.2016.11.012

Zlotta, A. R., Egawa, S., Pushkar, D., Govorov, A., Kimura, T., Kido, M., . . . van der Kwast, T. H. (2013). Prevalence of Prostate Cancer on Autopsy: Cross-Sectional Study on Unscreened Caucasian and Asian Men. *Jnci-Journal of the National Cancer Institute, 105*(14), 1050-1058. doi:10.1093/jnci/djt151